Patterning biomolecules on polymer surfaces for applications in life sciences

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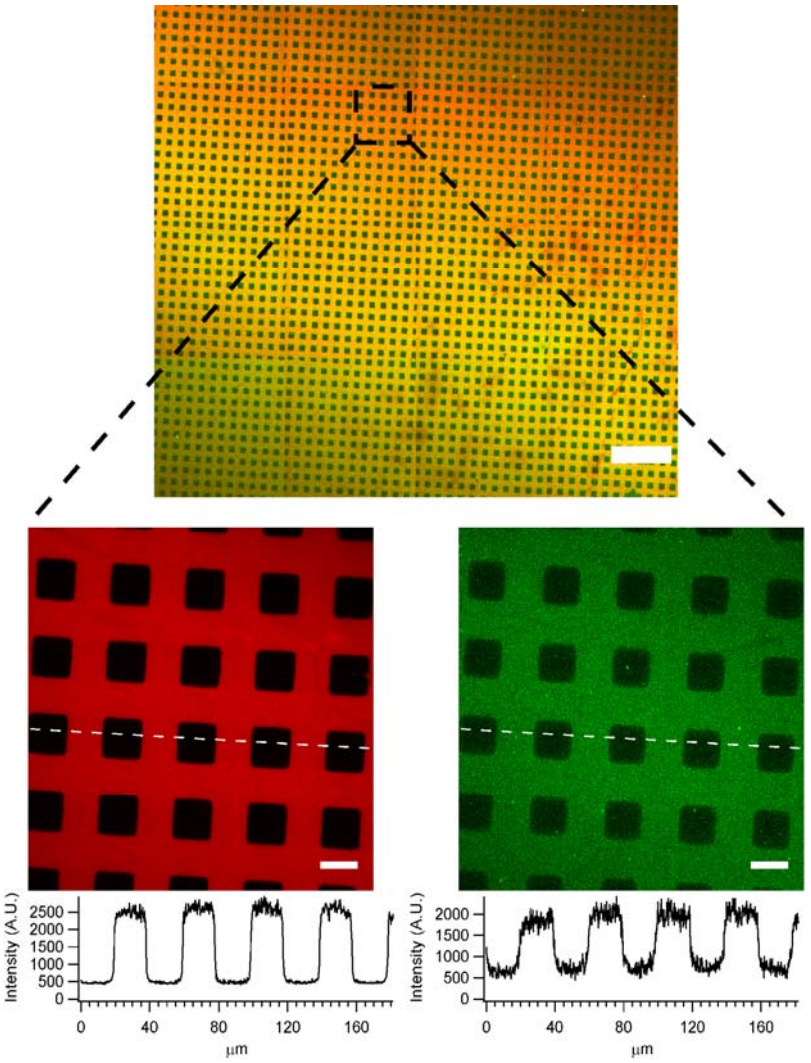
Susan Blak Nyrup



Risø National Laboratory
Roskilde
Denmark
December 2005

Per la luce della mia vita.

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Title: Patterning Biomolecules on Polymer Surfaces for Applications in Life Sciences.
Department: Danish Polymer Centre, Risø National Laboratory

This thesis is submitted to Graduate School of Biophysics, University of Copenhagen, Department of Chemistry in partial fulfilment of the requirements for the Ph.D. degree in biophysics.

Abstract :

The development of in-mould patterning, a novel technique for patterning proteins on polymer surfaces, is described. Patterns of proteins were transferred to polymer surfaces during polymer shaping by use of a simple and easily scalable process. In-mould patterning combines the industrial process of injection moulding with the patterning technique of micro contact printing. Through micro contact printing, proteins were transferred onto raised areas of a mould. The injection moulding process subsequently immobilized the proteins in defined regions at the polymer replica surface. Patterns with feature sizes spanning from nano- to centimetres were produced. Immobilization of proteins by use of this method was demonstrated for horseradish peroxidase, fibronectin, avidin and IgG. The structure of the transferred proteins was sufficiently preserved to retain their enzymatic activity, cell adhesion promoting properties, or ability to bind specific ligands, respectively.
As part of the development of the in-mould patterning technique, fundamental studies on micro contact printing using hydrophobic stamps were carried out.

To demonstrate the versatility of the technique, studies on Pd-colloid patterning were undertaken. Construction of interdigitated arrays of microscopic copper wires, located in recessed areas on polymer replicas through in-mould patterning was demonstrated. Pd-colloids were in-mould patterned onto recessed areas of a replica and subsequently served as catalysts for the electroless deposition of copper in the recessed areas.

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Cover : In-mould patterned fluorescently labelled avidin (red) specifically binding fluorescently labelled biotin (green).

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Contents

Preface

The work presented in this thesis was carried out at the Danish Polymer Centre, Risø National Laboratory in the period June 2002 to December 2005. Prof. Niels Bent Larsen, Risø National Laboratory and Prof. Kjeld Schaumburg, University of Copenhagen supervised the project.

One month was spent at University of Glasgow at the Centre for Cell Engineering led by professor Adam Curtis.

Two months were spent at Swiss Federal Institute of Technology at the Laboratory for Surface Science and Technology led by Marcus Textor.

All experiments presented were carried out by Susan Blak Nyrup unless otherwise stated.

1 Introduction and background

1.1 Protein-patterned surfaces for the life sciences

Development of techniques for immobilizing biomolecules such as oligonucleotides, DNA and proteins in specific predetermined positions on solid surfaces has attracted intense attention within the last years. This is due to the tremendously broad field of applications for bio-functionalized surfaces. Interests lie within areas such as fundamental research in cell biology[1,2], biosensor technology[3] drug discovery[4] and tissue engineering[5,6].

The development and commercialization of DNA microarray chips has revolutionized the field of genetics and is without any doubt the most well known development within biomolecular patterning. High-density arrays can comprise hundreds of thousands of spots of oligonucleotides or tens of thousands of cDNA probes per square centimetre. Consequently these arrays make it possible to detect thousands of different binding events in a single experiment and most importantly this can be done in a massively parallel manner requiring minute amounts of analyte[7,8]. DNA microarrays are being used for various purposes, but most prominently they are employed in locating disease related variations in DNA and in detecting variations in messenger RNA (mRNA) concentrations e.g. between cells at different stages of a disease. The thus obtained information on DNA variations and changes in mRNA concentrations is utilized within diagnostics and in the field of drug discovery[9].

It is, however, not the nucleic acids but the proteins that carry out practically all processes performed in cells. Therefore the encoded genetic information and its defects are ultimately displayed through their effect on the activity of proteins. Several studies have shown that there is a poor correlation between levels of mRNA and the levels of the corresponding active proteins[10]. One reason for this discrepancy is that turnover time and translation efficiency varies between different mRNA´s. In addition, most proteins require post-translational modifications, association with other proteins or phosphorylation in order to acquire their final active form[8]. Indeed the level of complexity is increased enormously when moving from nucleic acids to the proteome. This, along with the poor correlation between the mRNA profile and the proteome, necessitates investigation of proteins directly rather than through the nucleic acids, in order to study many of the complex processes involved in regulation of cell activity.

The development and optimization of protein microarrays will give access to extremely valuable specific information on protein-protein, enzyme-substrate and protein-small molecule interactions. This information will be of great importance in diagnostic applications. Moreover, as most drug targets are in fact proteins, access to such information will greatly increase the speed at which new drugs can be developed. It will allow more precise location of new possible targets, and

can simultaneously reveal undesirable cross reactivity[4,10]. Compared to the oligonucleotides that constitute the capture molecules of DNA-chips, proteins are vastly more complex. Proteins are inherently unstable and when removed from their natural environment many proteins do not retain their native functional state. Preservation of secondary and tertiary structure, post-translational modifications and complexes formed with other proteins are crucial to protein function. Therefore, ideally all these should be preserved throughout the patterning process[8]. These factors make patterning of proteins far more challenging than patterning of oligonucleotides and it significantly limits the possible immobilization methods. By now there are commercially available surfaces for covalent immobilization of proteins. These are integrated with detection systems for monitoring binding events and are being implemented in the screening of possible drugs[11]. Recent reviews on protein microarray technology emphasize, however, that several issues need to be addressed before protein microarrays can become a standard tool widely implemented in the biosciences and diagnostics. The arrays must provide reliable, quantitative and efficient results and for this to occur precise control over the ligand density must be achieved, background adsorption of proteins must be reduced and it may be necessary to implement oriented immobilization of ligands. Finally it is necessary that arrays reach a reasonable level of cost[4,8,9,12,13].

The combination of microfluidic systems and detection of analytes by use of surface-immobilized capture-proteins is another growing field employing protein-patterned surfaces. Traditional immunoassays such as Enzyme-Linked ImmunoSorbent Assays (ELISA) are performed in well-plates using large reaction volumes, typically 100 µl[14]. Therefore, they require long incubation times to reach adsorption equilibrium in each reaction step. The drastically reduced reaction volume used in a microfluidic system, typically a few nl, reduces the required incubation time from hours to minutes[15]. Thus these systems have the advantage of needing minute amounts of reagent and if they are employed in point-of-care diagnostics, these systems can provide fast answers needed e.g. when diagnosing myocardial injury[16].

Protein patterned surfaces are key elements also in biosensors[3]. This is clear from the definition of a biosensor as “a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element which is in direct contact with a transducer element”[17]. Many types of biosensors are by now commercially available and they are widely implemented in research laboratories, which is clearly illustrated by the over 1000 publications utilizing commercially available systems in 2004[18]. Biosensors are also used in medical diagnostics, environmental monitoring and the food processing industries. The development of biosensor systems is still actively pursued[17,19].

The presented examples do in no way represent a comprehensive list of applications. However, they illustrate that there are numerous applications for protein patterns on solid surfaces and many more applications are foreseen as

techniques for protein immobilization, prevention of unspecific adsorption and detection methods are developed further[4,9].

1.2 The potential of polymer materials in the life sciences

Glass and silicon are the most commonly used materials in fabrication of protein microarrays, biosensors and microfluidic systems for applications in the life sciences[11,13,20]. One practical reason for this is the convenience of being able to directly transfer many existing processing methods that have been developed, optimized and automated for microstructuring silicon in the semiconductor industry. Furthermore, commercial glass has no auto-fluorescence, which is a very significant quality, as one of the most commonly utilized methods for detecting captured ligands is through the use of fluorescence labelling and microscopy[8]. Finally a range of well-characterized chemical processes for derivatizing glass surfaces exist[21].

However, if protein microarrays, biosensors and microfluidic systems are to become part of the daily used tool box in biotechnology laboratories, or to be successfully implemented in e.g. point-of-care diagnostics, it is necessary to reach a reasonable product cost. Introduction of new production methods reducing device cost will therefore have a significant influence on the breadth of the fields in which these techniques will be implemented[7,13].

The high cost of processing glass and silicon makes it interesting to employ materials that offer simpler manufacturing processes and therefore lower end cost. Polymers possess a range of qualities that make them particularly appealing substrates and for this reason producers have already started to explore the possibility of substituting glass with polymers[21]. Material and processing costs are low, making the production of disposable articles feasible. This is particularly interesting for applications in medical diagnostics, as it eliminates the risk of cross contamination. Polymers are already widely used in traditional bioassays and as biomaterials[5] and polymer cell-culture substrates are the most widely used culture surfaces. This shows that polymers can be biocompatible, which is relevant in applications such as cellular arrays and cell-based biosensors. Furthermore a useful range of surface chemistry modifications is available, allowing the same basis-part to be optimized for various uses. This is probably best exemplified by the wide variety of all-polystyrene products with very different properties offered for use in cell culture. Finally the tremendously large variety of polymers allows optimization of material properties such as transparency, low auto fluorescence, mechanical properties and biodegradability[21].

1.3 Objective

As has hopefully been made clear in the previous sections, there are many good reasons to invest time and effort in developing methods for patterning proteins on solid surfaces. Certainly, drug discovery companies will invest large amounts of

money in order to exploit the possibilities offered by protein microarrays[22]. However applications within healthcare that could considerably speed up diagnosis and provide on-the spot answers are likely not to be implemented until device-cost are decreased. Therefore, there is a strong need for establishing fast and low-cost methods for forming protein-functionalized surfaces. This will assist the development and introduction of many of these exiting new products.

The main objective of this thesis was to develop a high throughput technique for patterned protein-functionalization of polymer surfaces.

Optimization and implementation of fundamentally new production techniques are often costly and therefore take many years to become fully implemented. We therefore opted to examine whether a high throughput technique could be developed on the basis of existing methods.

1.4 Existing methods for micropatterning proteins

This section provides an introduction to some of the existing techniques for micropatterning of proteins both on polymers and on other substrates. We have chosen to include processes on other substrates as many of the existing techniques for patterning polymer surfaces are derived from processes developed for e.g. glass. While it does not offer an exhaustive review of the field, this section should provide a general idea of the presently used approaches for protein patterning.

1.4.1 Photolithography

In photolithography, patterns are defined on a substrate using light. The substrate to be patterned is coated with a thin homogeneous layer of a UV-sensitive polymer, the photoresist. This is done by use of spin-coating. The photoresist is then hardened through evaporation of the solvent by placing the substrate on a hot-plate, typically at temperatures above 90^{O}C. The photoresist either becomes soluble after exposure to UV-light (positive photoresist) or becomes insoluble after exposure to UV-light (negative photoresist).

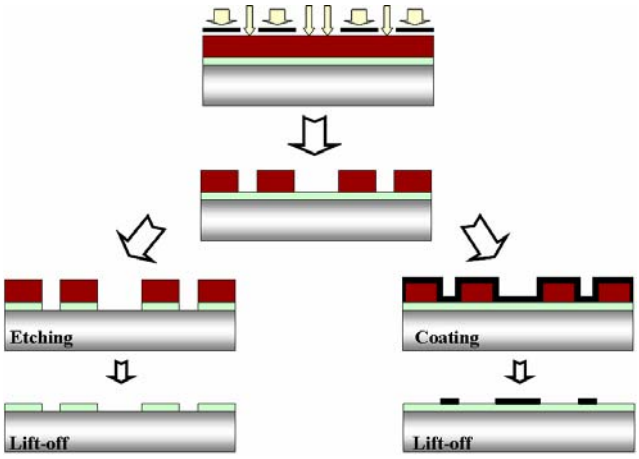


Figure 1 Schematic illustration of the photolithographic process when using a positive photoresist. A substrate spin-coated with photoresist (red) is exposed to UV-light through a shadowmask. The illuminated areas are subsequently dissolved in developer. The revealed substrate areas are either etched (left) or coated/chemically modified (right). Remaining resist is removed by lift off using organic solvent.

Consequently patterns can be formed in the photoresist by illuminating it in specific areas. This is done by use of a shadowmask, generally consisting of a glass plate with the desired pattern defined in non-transparent chromium. After illumination, the pattern is developed by dissolving solubilized photoresist. This exposes the underlying substrate in selected areas, while photoresist provides protection of the remaining coated areas. This permits chemical modification, deposition of material or removal of substrate material selectively in the exposed substrate areas. After modification, the remaining part of the photoresist is removed by lift-off i.e. dissolution in an organic solvent, see Figure 1.

As the semiconductor industry has been the main driving force for developing the photolithographic technique, materials used in the process are also optimized for this purpose. Due to the fragile nature of proteins it is not generally feasible to use traditional photoresists for patterning proteins. The alkaline developing solution and the organic solvent (typically acetone) involved in the lift-off is likely to denature most proteins. The elevated hardening temperatures and the use of organic solvents also prevent the use of "traditional" photolithography for protein patterning on polymer surfaces, as e.g. polystyrene is dissolvable in acetone. However, several groups have recently presented photoresists requiring only low-temperature hardening and mild developing solutions such as Phosphate Buffered Saline (PBS)[23,24]. This opens up the possibility of performing photolithography on polymer substrates. However, one concern remains with the technique for using these photoresists. Prior to lift off the remaining resist is rendered soluble by a final short UV-exposure. Proteins patterned after the first exposure and development will thus be exposed to UV-light in the final exposure of the entire substrate surface. This may influence protein function by causing oxidation and cross-linking within the protein structure. Despite this concern these developments show great promise for protein patterning on planar substrates.

1.4.2 Micro contact printing

Patterning by micro contact printing is often compared to using a regular office stamp or, for the more playful, to doing potato printing, which very well captures the straightforwardness of the technique.

Micro contact printing, developed by Whitesides and co-workers[25], utilizes a topographically structured elastomeric stamp. Such stamps are formed by casting a liquid phase pre-polymer on a topographically structured master. Typically stamps are produced from polydimethylsiloxane (PDMS). Polymerization of the

pre-polymer is obtained by curing, and after this the stamp is gently separated from the master, see Figure 2.

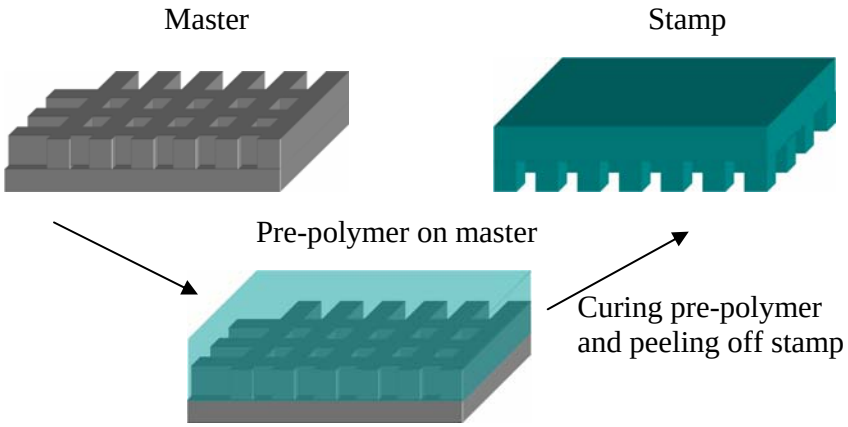


Figure 2 Schematic illustration of the stamp formation process.

After being separated from the master, stamps can be utilized without further treatment[26,27]. However many groups choose to modify the stamp surface in order to achieve a hydrophilic surface that is more easily wetted by aqueous solutions[28,29]. This is typically done by exposing the stamp to an air or oxygen plasma.

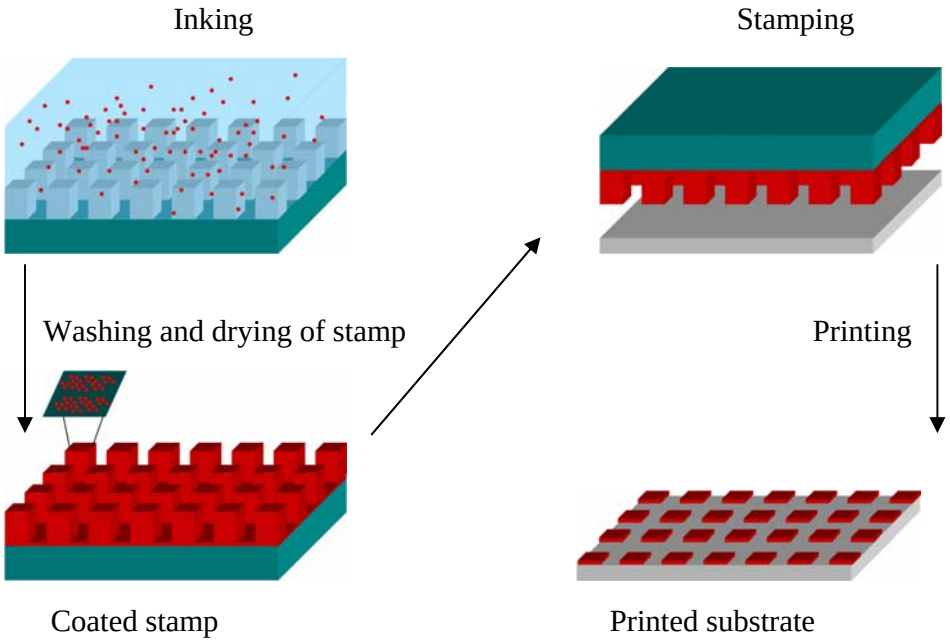


Figure 3 Schematic illustration of the inking and printing process in micro contact printing.

The stamp is “inked” by incubation in a solution containing the molecules to be printed. After a suitable incubation time, 1 - 45 min depending on the molecules to be printed, the stamp is dried and placed on the substrate. Conformal contact is formed between the substrate and the protruding stamp-structures and in the contact regions molecules are transferred to the substrate, see Figure 3.

Once a master structure is available, the stamp production can be carried out in a couple of hours. Stamp materials are inexpensive and stamp production; inking and printing can be carried out in a regular laboratory without the need for clean room access. These factors make the technique inexpensive, very flexible, fast and accessible to most laboratories, which certainly has contributed to the popularity of the technique.

A wide range of materials as e.g. proteins, catalysts, colloidal particles, polymers and several types of alkanethiols can be printed. The latter are utilized for patterning proteins and cell adhesion[2]. When printed on e.g. a gold surface, hydrophobic alkanethiols form self-assembled monolayers that are covalently bound to the surface. Backfilling the bare regions with protein resistant polyethylene glycol-terminated thiols forms a pattern of protein-permissive and non-permissive areas. Consequently incubating the surfaces in a protein solution causes formation of protein patterns[30].

While this system requires a gold (or silver or copper[31]) surface, direct printing of proteins can be performed on glasses, silicon and polymer surfaces. From the literature it would seem that micro contact printing on unmodified polymer surfaces is not a straightforward task[32]. However several groups have developed methods for patterning all-polymeric substrates. Chilkoti and co-workers developed a technique termed Microstamping on an Activated Polymer Surface (MAPS)[33-35]. Reactive sites were introduced on polymer surfaces. Through these sites proteins presenting amine groups could be covalently linked to the surface. Derivatization was performed on the entire surface and the patterned immobilization of proteins was achieved by use of micro contact printing. This was demonstrated for polystyrene, polymethylmethacrylate, polyethylene and polyethyleneterephtalate. The wide range of possible substrates makes this technique interesting. However, the use of organic solvents in the derivatization process may introduce toxic substances in the substrate that may later be released. This is problematic in particular for the production of cell culturing substrates.

Another elegant way of creating both topographical and chemical patterns exploits the reorientation of a comb polymer containing oligo(ethylene glycol) upon exposure to water[36]. The amphiphilic polymer is soluble in water and ethanol and can be spincast or micro contact printed onto a wide range of polymer surfaces, after which it is no longer soluble in water. Prior to exposure to water the polymer can be patterned with proteins by micro contact printing. Upon exposure to water, areas of the polymer that have not been patterned with proteins reorient to present the oligo(ethylene glycol) combs rendering these areas highly protein and cell resistant.

Finally, less complicated surface modifications; such as plasma treatment can also render polymeric substrates “printable”[37].

Due to the elastomeric nature of the stamp it is necessary to pay careful attention when designing the stamp pattern. Structures of high aspect ratio (height of

structure/width of structure) are unstable and thus lead to poor pattern fidelity. Concurrently large separations between low structures cause recessed regions of the stamp to sag and make unwanted contact with the substrate[38]. However, new stamp materials[39,40] and printing strategies[41] have expanded the range of possible feature sizes, demonstrating formation of patterns with feature sizes from 80 nm up to several mm[39]. Micro contact printing thus offers the opportunity to pattern polymer surfaces, create patterns over large areas and do so with an extremely wide range of possible feature sizes. Furthermore it is possible to create patterns with a high degree of complexity. This flexibility makes micro contact printing an extremely interesting patterning technique.

1.4.3 Homogeneous coating-processes combined with photolithography, masking, laser ablation or micro contact printing

Plasma polymerization and chemical vapour deposition are techniques that can be employed in forming homogeneous thin polymer films on topographically structured surfaces. A plasma is a highly ionized gas, which can be generated by application of high-frequency electric fields causing electric discharge. Plasma polymerization takes advantage of the highly reactive molecular species created in plasmas. Gaseous monomers are introduced in the plasma where they are activated or fragmented. This can be utilized to initiate polymerization on a substrate placed within the plasma chamber. Another interesting method for polymer film deposition is Chemical Vapour Deposition (CVD). With this technique films are deposited by exposing the substrates to volatile precursor molecules that react to form the desired coating. Both methods can be used to coat large surface areas and are applicable to a wide range of substrates including polymers.

Using these methods it is possible to generate both coatings that are highly protein resistant and coatings that are permissive to protein adsorption. This can be exploited for protein patterning in several ways. Employing photolithography, part of a substrate can be masked off with resist prior to polymerization. Upon lift-off uncoated areas will be revealed and if the utilized substrate is permissive to protein adsorption and the deposited polymer is protein resistant, adsorption will occur solely in un-patterned areas. Alternatively the substrate can be coated homogeneously prior to the photolithography step and subsequently be taken through the described process which e.g. Hoffman and co-workers have demonstrated[42,43]. Masking of the surface can also be achieved using a physical mask that is applied to the surface prior to polymerization and is peeled off afterwards[44]. Finally polymerized material can be removed by laser ablation[45] thus revealing the underlying substrate.

Proteins can also be patterned on homogeneous protein-resistant coatings by use of micro contact printing. Using CVD, Langer and co-workers produced two types of coatings that, without further activation steps, formed covalent bonds with an amino-terminated biotin ligand[46,47]. The biotin ligand was patterned on

the surface by micro contact printing. After micro contact printing they demonstrated selective binding of first avidin and subsequently biotinylized antibodies in the patterned areas. These antibodies were used to induce adherence of endothelial cells solely within the patterned regions. However, no study of the long-term properties (more than 24 hours) of these coatings with regards to resisting unspecific protein adsorption and constraining cells within the defined patterns has been presented.

Special care needs to be taken to optimize deposition parameters in order to prevent delamination of the produced films. However, the wide variety of substrates that can be patterned, the many possible variations in coatings that can be made and the possibility of coating large areas make plasma polymerization and CVD attractive candidates for large-scale production[47]. Deposition of "stampable" protein-resistant coatings is also an appealing approach for patterning as this solves the problem of unspecific protein adsorption that plagues many surfaces. Furthermore the process does not involve use of organic solvents and therefore there is low risk of introducing toxic substances into the polymer matrix during the process.

1.4.4 Photoimmobilization

Immobilization of proteins through photoreactive groups can be performed on many types of substrates including polymers[48,49]. The most attractive feature of photoimmobilization is the opportunity to pattern proteins in recessed areas and enclosed volumes. Holden *et al.* recently presented a strategy for patterning multiple types of proteins within microfluidic channels filled with aqueous solution at near neutral pH. Channels are coated with a blocking protein and rinsed. Subsequently fluorescence labelled biotin is introduced and by locally exposing the channel to 488 nm laser light, the fluorophore moieties are bleached. This creates short-lived reactive species that form covalent bonds with the blocking-proteins deposited on the channel walls. Subsequently the biotin can be utilized to immobilize first avidin and then biotinylized proteins. By performing two such patterning sequences they demonstrated patterning and retained function of two spatially separated patches of enzyme. The wide range of substrates on which this technique can be used and the opportunity to pattern proteins sequentially in non-denaturing conditions makes this an appealing technique.

1.4.5 Dip-pen nanolithography

Dip-pen nanolithography (DPN) was developed in 1999 by Mirkin and co-workers[50]. In short the technique utilizes an Atomic Force Microscopy tip coated with the molecules that are to be immobilized. When the tip is brought into contact with a surface, humidity from the air creates a meniscus between the surface and the tip and in this meniscus proteins are transported off the tip onto the substrate, see Figure 4

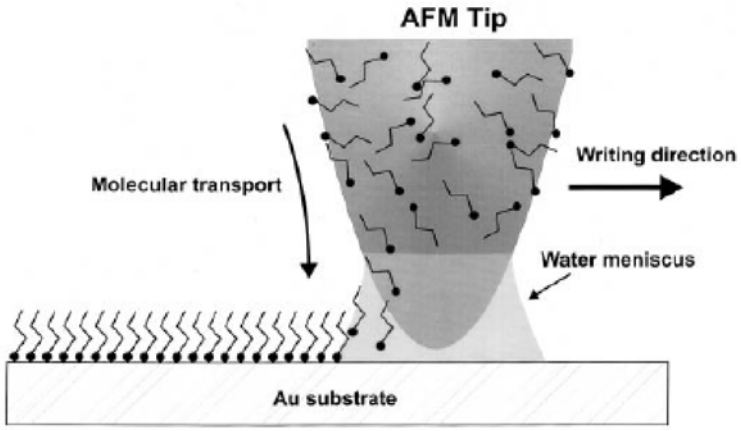


Figure 4 Schematic illustration of the dip-pen nanolithography process. Reprinted with permission from[50]. Copyright 1999 AAAS.

Increasing or decreasing the contact time of the tip and varying the humidity, produces variations in feature size[50]. Protein patterns with feature sizes below 100 nm have been created using DPN[51] and the technique can be utilized to pattern proteins in registration with nanometre range precision[52]. This, to our knowledge, is not achievable using any other technique. DPN is slow compared to e.g. spotting (see section 1.4.6), but provides the possibility of creating very high-density arrays and the option of controlled application of protein on e.g. mass sensors. Progress has been made towards parallel writing and individual inking of tips in large arrays[52]. However, the low writing speed makes the technique poorly suited for coating of larger areas, which may e.g. be necessary when creating cellular arrays.

1.4.6 Spotting

Printing small drops of molecules onto a surface is the established method for producing high-density protein microarrays[53]. There are several commercially available printing systems that can be classified as either contact or non-contact printing systems. In contact printing systems micromachined pins are brought into physical contact with the substrate to be patterned. Non-contact printing technologies make use of piezoelectric and ink-jet technology for dispensing solution. Depending on the utilized surface, proteins are immobilized either by physical adsorption, affinity immobilization e.g. using the avidin-biotin system or by covalent coupling to a functionalized surface[13,54]. The smallest feature size possible is below 250 nm[55]. However, this is produced using a system that does not offer printing of multiple proteins at one time, which will considerably slow down production of arrays.

1.4.7 Micro-fluidic patterning

Microfluidic patterning utilizes adsorption of proteins from a liquid that flows in a microfluidic network sealed on the surface to be patterned. Channels in the network are typically between 1 and 100 μm and the most frequently used material for forming these networks is PDMS. PDMS is an advantageous material because of the conformal sealing it forms with most substrates. When patterning by use of microfluidic networks the possible patterns that can be formed are restricted by the continuous nature of the channels. However, using microfluidic networks, concentration gradients, which are relevant in studies of chemotaxis, can be formed on the exposed substrates. This is not easily achieved using other techniques[56].

A strongpoint of microfluidic systems is the ability to simultaneously pattern several different proteins in separated regions of a surface. This can either be done by exploiting the laminar flow-pattern within microchannels or simply by flowing different protein solutions through separate channels in the network. This was utilized by Bernard *et al*. to form immunoassays presenting several different antibodies for screening of analyte solutions[57]. A flat PDMS surface was sealed against a multi-channel microfluidic network formed in silicon. Several different antibodies were then adsorbed on the PDMS by flowing a different antibody solution through each channel. After this the entire PDMS surface was blocked using human serum albumin. A second set of microfluidic channels were sealed on the surface such that the channels were now perpendicular to the lines of antibodies deposited in the first step. This allowed an analyte solution to be interrogated against several antibodies in a single-step.

A method for forming isolated structures from solution was presented by Hyun *et al*.[58]. An array of microwells was formed in PDMS and the surface was rendered hydrophilic by plasma treatment. Subsequently raised areas between the wells were rendered hydrophobic by micro contact printing with hexadecanethiol using a flat stamp. This eased the filling of wells with protein solution. Hereafter the substrate to be patterned was placed on the well-array and by inverting the structure, substrate areas located over wells was brought in contact with the solution. While it is not a microfluidic network, the technique offers similar advantages such as immobilization of several proteins in separated areas in a single step. Depending on the pre-treatment of the polymer substrate used, proteins can be physisorbed or attached covalently to the exposed surface areas using both well patterning and microfluidic networks.

The presented protein patterning methods offer many possibilities for formation of both continuous line patterns and isolated structures We chose to focus on micro contact printing as it is a versatile technique, allowing formation of complex patterns over large areas. Moreover protein transfer occurs within seconds making the patterning step very fast compared to e.g. photolithography or DPN.

2 Introduction to micro contact printing

2.1 The short story

Micro contact printing was invented by Whitesides and co-workers in 1993[25]. Initially the technique was introduced as a method for patterning etch-masks of self-assembled monolayers (SAMs) of hexadecanethiols on gold surfaces. Already in 1994 the method was expanded to applications within protein and cell patterning. Singhvi *et al.* printed patterns of hexadecanethiols on gold surfaces and followed the printing step with a backfill of polyethylene glycol-terminated thiols[2]. Polyethylene glycol-terminated thiols resist protein adsorption whereas the hydrophobic hexadecanethiols support protein adsorption. The achieved patterning could therefore be utilized to preferentially adsorb cell-adhesion promoting extracellular matrix proteins that promote cell adhesion on the hexadecanethiol regions. This allowed patterned adhesion and restricted spreading of cells that were cultured on the surfaces after the protein adsorption. Patterning of SAMs prepared as described is probably still the best-described micro contact printing system and it has been used extensively to study cell interactions at the single cell level[1,2,59,60]. For details and figures illustrating the stamp formation and printing procedure see section 1.4.2.

In 1998 Bernard *et al.* expanded the technique to the direct patterning of proteins[26]. They found that when monolayer quantities of protein were adsorbed on a stamp surface these could, after rinsing and drying the stamp, be printed onto a variety of substrates. Tested substrates included silanized glass, gold, silicon, silicon oxide, polymethylmethacrylate and polystyrene[27]. Proteins were transferred with an efficiency of more than 99 %. Most importantly, they found that for a wide range of proteins there was little or no noticeable difference between protein function of the printed proteins and proteins adsorbed from solution.

Micro contact printing has become an extensively used technique within protein and cell patterning, resulting in a steady stream of publications presenting either results produced through use of micro contact printing or additional developments of the technique. The following section gives an introduction to micro contact printing of proteins and some of the advances that have been made in the field since the introduction of the technique.

2.2 The making of master and stamp

2.2.1 Master structures

Master structures for moulding of stamps are typically created through photolithographic processes (see Figure 1 section 1.4.1). However, masters with feature sizes below 1 μm require the use of e-beam lithography followed by reactive ion etching. E-beam lithography works in much the same way as photolithography; only here the resist material is electron sensitive instead of UV-sensitive. Exposure of the resist is performed by direct writing with an electron beam and therefore no shadowmask is required for e-beam lithography.

2.2.2 Stamps - design and materials

The elastomeric nature of the stamp is both the blessing and the curse of micro contact printing. When a PDMS stamp is placed on a substrate surface, the contact between stamp and substrate is initiated at single protruding points and then propagates across the interface. This gradual contact formation is important as it lowers the risk of trapping air under the stamp and it is possible only because the stamp is flexible and thus can adapt to height variations over long distances (see Figure 5 left part).

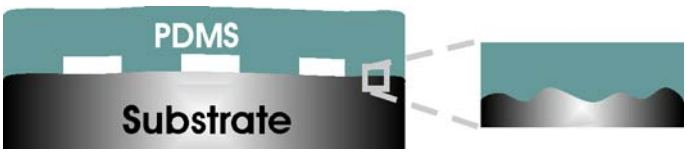


Figure 5 Conformal contact requires adaptation to height variations over long distances (left) and to small-scale roughness of the surface (right).

Elasticity of the stamp material is also essential in order to permit conformal contact i.e. contact at the molecular level, between the stamp and the substrate surface (Figure 5 right). This intimate contact is a prerequisite for transfer of the proteins[41]. The ability to form conformal contact is therefore one of the fundamental requirements to stamp materials if micro contact printing is to be applicable, not only to atomically flat surfaces.

On the other hand the elasticity of the stamp can lead to poor pattern fidelity owing to sagging of recessed areas, lateral instability of high aspect ratio features and neighbouring structures “sticking” to each other[41] see Figure 6.

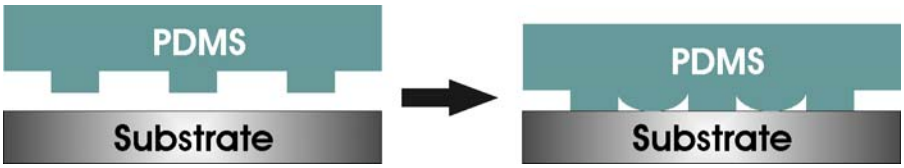


Figure 6 Schematic illustration of sagging between stamp features.

Delamarche *et al*. examined the stability of structures formed using Sylgard 184 from Dow Corning, the PDMS most commonly used for printing[61]. They found

that the aspect ratio (see p. 111) of protruding features must be below 2 to ensure lateral stability, while recessed areas must have an aspect ratio above 0.2 to prevent sagging. This severely limits the possibility of making small structures separated by large distances. Several strategies have successfully been pursued in order to enable printing of smaller features with larger separation. A few are listed here.

New stamp materials with lower elasticity have been proposed by several groups[39,40]. Csucs *et al*. suggested the use of polyolefins, a relatively new class of polymers[40]. As opposed to PDMS, polyolefins are thermoplastic. Therefore, the authors produced stamps by melting a block of the polymer on top of the master while applying pressure (Figure 7).

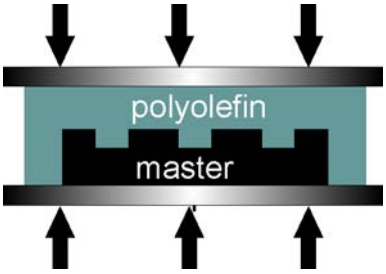


Figure 7 Schematic illustration of the formation of polyolefin stamps

The resulting stamps contained a group of 100 nm wide lines separated by 3 μm. Printing was performed using Sylgard 184 PDMS and polyolefin stamps made from the same master. Using the polyolefin stamps defect-free 100 nm lines of fluorescence labelled protein were printed. No sagging occurred even in areas where two sets of lines were separated by a 12 μm gap. Using PDMS stamps, the produced 100 nm lines were extremely distorted and sagging had occurred in the 12 μm gap. It is therefore clear, that using materials of lower elasticity can significantly improve stamp performance.

Michel *et al*. proposed the use of siloxanes with lower elasticity and these showed considerably improved stability relative to Sylgard 184[39]. To further increase the stamp stability they proposed to construct stamps of several layers of varying stiffness. They found that the best results were achieved by using two-layer stamps consisting of a thin flexible glass backplane (100 μm) and a thin siloxane stamp (75 μm) of low elasticity see Figure 8. The more rigid backplane provided added stability to the stamp, thus lowering the risk of sagging. At the same time the flexibility of the backplane allowed the contact line between stamp and substrate to form gradually as needed for formation of defect free contact between the stamp and the substrate. Using this strategy the authors were able to print 100 nm features over 5 x 5 cm large areas.

Figure 8 Stamps reinforced with glass backplane.

Michel *et al*. proposed another printing strategy, termed subtractive inking[41]. First a flat Sylgard 184 stamp was homogeneously coated. Then a microstructured silicon surface was placed on the stamp, thus "subtracting" ink in the areas of contact. Subsequently the remaining protein pattern on the stamps was transferred onto a substrate by regular printing. This simple approach allowed formation of feature sizes down to 100-200 nm. Indeed, depending on the feature sizes needed, there are several options for allowing conformal contact while minimizing the risk of sagging and instabilities.

Prior to the printing it is necessary to ink the stamp, and after placing the stamp in contact with the substrate, proteins must be able to transfer off the stamp onto the substrate. These two processes can be modulated through various surface treatments of the stamp.

2.2.3 Surface modifications - optimizing the inking of stamps

It has been reported that the use of unmodified PDMS stamps produces highly non-uniform patterns[28]. This was ascribed to poor wetting of the hydrophobic stamp by water-based inking solutions. However, several groups have presented homogeneous patterns produced by micro contact printing using hydrophobic stamps[27,62-64], thus demonstrating that it is indeed possible to use unmodified stamps. Many groups however opt to modify stamp surfaces prior to inking[33,47].

The most commonly used modification procedure is to hydrophilize stamps using oxygen plasma treatment. This increases the oxygen content in the stamp surface thus producing a more hydrophilic surface[65]. Morra *et al*. studied the hydrophobic recovery of plasma treated PDMS and found that within 3.5 hours after plasma treatment the advancing contact angle changed from 30° to 68° when stamps were stored in air[65]. The advancing contact angle of untreated PDMS was 108°. They also found that if stamps were placed in water after plasma treatment the hydrophilicity was retained. However Lawton *et al*. found that when they examined contact angles under the conditions they used for micro contact printing, a similar hydrophobic recovery occurred for stamps stored in air and in water[66]. To avoid variations in printing results related to variations in the hydrophilicity of the stamp, it is therefore important to use a standardized protocol when stamps are modified using plasma treatment.

Several other strategies have been developed to optimize coating of stamps for various purposes. Bernard *et al*. developed a particularly interesting technique, called affinity printing[67]. Antibodies were covalently coupled to the surface of PDMS stamps and retained the ability to specifically bind their ligands. Stamps could therefore be immersed in complex protein solutions and still bind only the desired ligand on the surface. Subsequently stamps were rinsed in water, dried and placed on the substrate surface, and when released only the ligand molecules remained on the substrate surface. The affinity-coated stamps could be used at least 10 times. By covalently coupling different antibodies in separate areas of

the stamps, the authors were able to ink stamps with different ligands in a one step inking process, see Figure 9.

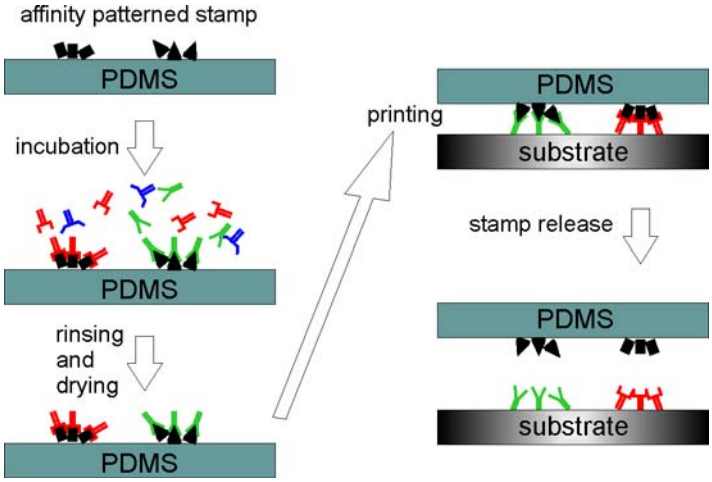


Figure 9 Schematic illustration of affinity printing. Figure adapted from[68]

The functionalized stamps were simply dipped into a solution containing a mixture of the ligand molecules. These stamps allowed substrates to be patterned with several different proteins in registration and in one printing step. A capacity that is certainly interesting when considering applications such as protein arrays.

Finally Delamarche *et al.*[62] further developed the strategy of using flat stamps for printing by devising two methods for patterned coating of flat stamps. Both of these methods make use of a flat PDMS stamp patterned with polyethyleneoxidesilane (PEO-Si). This patterning is achieved by exposing the stamp surface to an O_2 plasma, while masking off part of the surface with a metallic mask. PEO-Si can subsequently be grafted in the oxidized regions, see Figure 10.

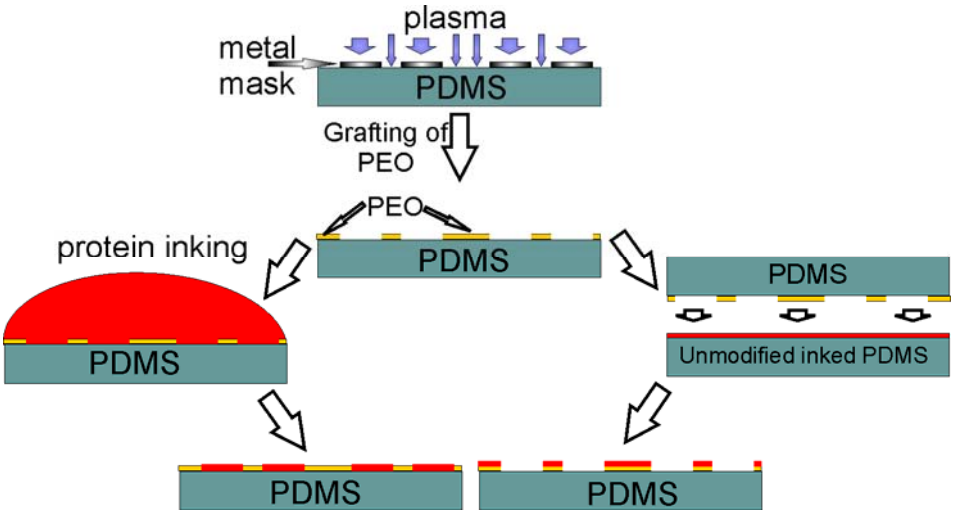


Figure 10 Schematic illustration of selective inking of unmodified stamp areas (left) and contact-inking of PEO grafted areas (right).

In the first method a patterned coating is achieved by incubating the modified stamp in protein solution. The protein repellent nature of the grafted PEO-Si causes adhesion selectively in the unmodified areas of the stamp, thus forming a patterned coating that can be printed onto a substrate surface. The second method utilizes the preferential transfer of proteins to more hydrophilic surfaces. If an unmodified protein coated stamp is printed on an unmodified stamp, proteins are not transferred from one stamp to the other. However proteins can be transferred from an unmodified stamp to PEO grafted areas. This can be utilized to ink the PEO-Si grafted regions of the flat stamps. A flat unmodified stamp is coated and when brought into contact with the modified stamp, proteins transfer only onto the PEO-modified regions. After this contact-inking step, proteins can be transferred from the PEO-Si to a glass substrate surface. If this method is combined with a patterned inking of the inker-pad through use of microfluidic networks, this approach too allows patterning of more than one kind of proteins on a surface in a single printing step.

2.2.4 Transfer

The mechanism by which proteins are transferred from the stamp to the substrate is not clear[27,32]. Yet, from experimental results, it is clear that the mechanism differs from the mechanism that causes proteins to adhere to surfaces in solution. It is also clear that the printing process is greatly influenced by the presence of water. Proteins can be micro contact printed onto surfaces that, when exposed to proteins in solution, are strongly protein resistant, such as e.g. surfaces with coatings presenting oligoethylene glycol[32,36,62]. Proteins remain immobilized on these surfaces even when exposed to aqueous media. Conversely, surfaces that proteins readily adsorb on from solution, such as e.g. polystyrene that is used in ELISA for exactly this reason, are not straightforward to pattern by micro contact printing[26,32]. Finally if printing is performed in water, no transfer occurs and if stamps are dried excessively prior to printing, the transfer efficiency decreases[26]. The necessary properties of substrate surfaces, in order for efficient transfer to take place, will be discussed in section 3.7.

3 Micro contact printing – experimental section

3.1 To modify or not to modify stamps

As our primary interest was to examine whether micro contact printing could be integrated in a high throughput production of protein-patterned surfaces, we decided to investigate micro contact printing using unmodified stamps. We chose to do so since most of the reported methods for modifying stamps do not produce surfaces that are long-term stable[65,66,69]. Protein adsorption varies depending on the surface properties of the stamp. Therefore change in the surface coatings such as hydrophobic recovery would be likely to cause problems with reproducibility. In a production, one could in principle choose to repeatedly plasma treat stamps prior to each inking cycle, however continually carrying out a process that degrades the printing surface used does not seem an appealing approach, particularly if one is attempting to produce patterns with feature sizes in the micro- or sub-micrometer range. All stamps were therefore utilized without any surface modification. We chose to use Sylgard 184 (from Dow Corning) as this is the most studied stamp material. Structured stamps were prepared against silicon masters with a relief pattern made in photoresist (SU-8 from Microposit). These masters were kindly prepared by Prof. Niels Bent Larsen. Flat stamps were prepared by casting and curing against polystyrene petridishes (NUNC).

3.2 Preparing stamps for printing

For the main part of the presented applications (section 1.1) it is important that the protein distribution within the stamped pattern is homogeneous. In the case of devices aimed at cell culture arrays it is well known that variations in protein concentration can influence cell adhesion and motility, causing e.g. directed migration of cells on the surface (chemotaxis)[70]. However, if the main function of the transferred protein is to promote cell adherence and if minor protein concentration variations do not modify cell performance, some inhomogeneity in the printed protein patterns is acceptable. This is not the case when preparing analytical devices such as e.g. protein chips and biosensors. For these, strict control of the presented protein concentration is required as this is critical to producing quantitative results.

To achieve homogeneous protein patterning by use of micro contact printing, proteins must first adhere to the stamp creating a homogeneous layer. Then the stamp must be dried in a way that does not redistribute the proteins, and finally the protein layer must be completely transferred to the desired surface again without redistribution of the proteins. Many different unintended protein patterns have been observed on both inked stamps and micro contact printed surfaces

during the course of this work. The variety of these patterns and the conditions under which they occur show that great care must be taken in each step of the micro contact printing to obtain the desired homogeneous protein coatings. In the following sections some of the observed unintended patterns, their possible causes and in some cases ways of avoiding them will be presented.

3.3 Cleaning and storing stamps

After curing PDMS on the desired master structures, a commonly reported procedure for cleaning stamps prior to protein coating is sonication in a 1:1 solution of ethanol and water[32]. This procedure was used initially and to ensure that no ethanol was left in the stamps, when protein solution was applied, stamps were stored in water prior to inking. Stamps were sonicated in a 1:1 solution of ethanol and water for 15 min and thereafter stored in Millipore® water at 4 °C for 2 days. To facilitate direct imaging of proteins adsorbed on the stamps and transferred to the substrates, we employed a fluorescence labelled antibody, Alexa Fluor 546 goat anti-mouse IgG (H+L) from Molecular Probes. These proteins will hereafter be referred to as IgG-Alexa. Unless otherwise noted microscopy images were recorded using a LSM5 Pascal (Carl Zeiss, Oberkochen, Germany) confocal laser scanning microscope. When recording fluorescence images, fluorophores were excited using the 488 nm or 543 nm laser lines of the scanning system. Flat stamps (app. 0.5 cm^2) were inked by placing a 90 μl drop of a 2 μg/ml solution of IgG-Alexa on the stamp surface. After 1 min incubation at ambient conditions, the protein solution and loosely bound protein was rinsed off by immersing stamps in a large volume of Millipore® water. The stamps were then dried using a gentle flow of argon and immediately placed on a cleaned microscope slide. All glass and silicon surfaces utilized were cleaned beforehand by 1 min immersion in chromesulphuric acid followed by rinsing in a 1M solution of KOH and finally rinsing in copious amounts of water. The stamps were placed such that only half of the stamp was in contact with the glass substrate. This allowed evaluation of both coating homogeneity and efficiency of transfer.

Fluorescence microscopy images of a printed glass substrate and the stamp that was used to print it are shown in Figure 11 A and B. Only the leftmost part of the stamp has been in contact with the glass substrate, the partial transferral of proteins rendering the stamp darker in this area. Figure 11 B shows the printed glass surfaces.

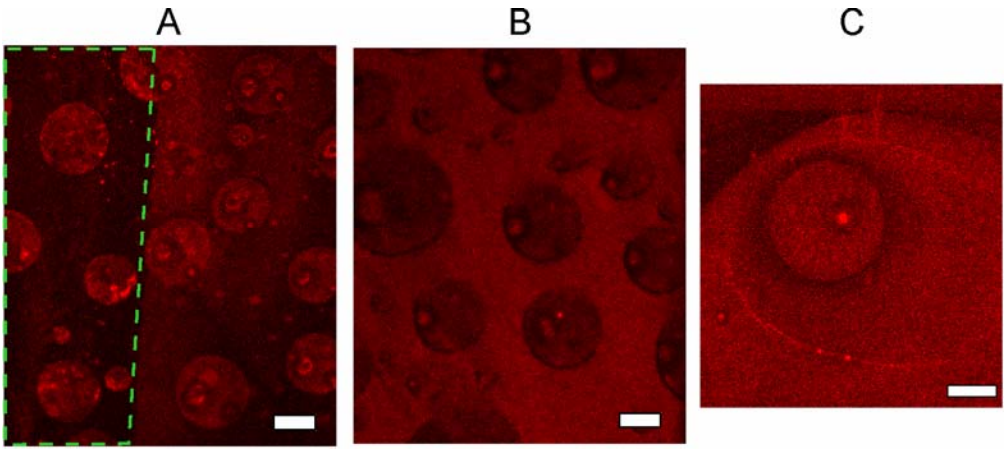


Figure 11 Fluorescence microscopy images of IgG-Alexa on: (A) A stamp inked after 2 days storage in water. The dashed green line indicates the border of the area that has been placed in contact with a glass substrate (B) The printed glass substrate. (C) A stamp on which a water drop has been dried during the inking process. Scale bars are 20 μm (A, B) and 100 μm (C) .

Circular areas with higher protein concentration are seen both in areas that have been in contact with the glass and in areas that have not. These structures can therefore not be the result of a patterned partial transfer of proteins during printing but must have been formed during the inking and drying of the stamp. When inspecting stamps after storage in water it was found that they were no longer transparent but looked foggy. When removed from water and left in air stamps slowly returned to being transparent. Figure 12 shows an image of a flat PDMS stamp stored in water for 5 days and removed from the water 5 min prior to the recording of the image.

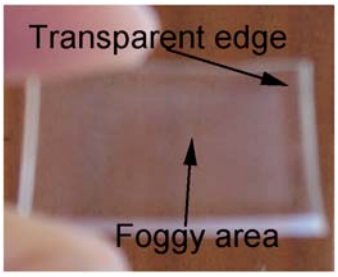


Figure 12 Flat stamp after storage in water. The edge of the stamp has returned to being transparent whereas the central part is still foggy.

When heated 2 min at 100°C the stamps become transparent, and upon immediate cooling return to being foggy. This strongly indicates that the foggy appearance is due to water droplets within the stamp. Contact angle measurements showed advancing and receding contact angles of 94° and 68° respectively for stamps stored in water and 120° and 109° for stamps stored in air. The increased inhomogeneity of the surface, indicated by the increased difference in advancing and receding angles, and the lower hydrophobicity

suggest that the surface contains areas of varying hydrophobicity. This is likely to be the cause of the inhomogeneous coating and poor transfer observed when using these stamps, as it could affect the coating process of the stamp in several ways. Figure 11 C shows a fluorescence microscopy image of a flat stamp coated with IgG-Alexa in the same way as described above. The stamp was not stored in water prior to the inking. During the drying of the stamp a droplet of water remained on the stamp surface and was dried out there, rather than removed with the argon flow. This produced a protein distribution similar to what was observed for stamps stored in water. If areas of higher hydrophilicity are present on the surface of stamps stored in water, this could cause retention of water droplets on the surface during drying, thus causing drops to dry out on the surface creating the observed intensity variations. In addition, for complete transfer of proteins from PDMS to occur, the surface of the stamp must be dry[27]. As illustrated previously, the outer rim of stamps, that have been stored in water, becomes transparent within a few minutes after removal from water. During this period water will be released at the surface of the stamp, and this could be the cause of the observed poor protein transfer.

Stamps were therefore not stored in water in the remaining experiments presented.

3.4 Protein redistribution by crystal formation

Solutions for inking were prepared by diluting a purchased high concentration protein solution in either Dulbecco´s-Phosphate Buffered Saline (D-PBS) or Phosphate Buffered Saline (PBS) (Invitrogen). The solution therefore contains a high concentration of different salts (app. 11 mg/ml). As the conformation of proteins is affected by solvent alterations it would be preferable to avoid rinsing the inked stamp in pure water. However, if the stamp is rinsed in PBS between inking and drying, salt crystals may form on the surface of the stamp. We performed a series of experiments to determine whether washing in PBS instead of water was feasible. Flat stamps (app. 0.5 cm^2) were inked by placing a 90 μl drop of a 50 μg/ml solution of IgG-Alexa on the stamp surface. After 10 min incubation the protein solution and loosely bound protein was rinsed off by immersing the stamps in a large volume of PBS. The stamps were then dried using a gentle flow of argon.

Bright-field microscopy images of the stamp surface revealed areas covered by salt crystals and fluorescence microscopy images in these areas showed a highly inhomogeneous protein coating. The inhomogeneity coincided with the position of the crystals showing a higher fluorescence intensity along the outer rim of the salt crystals, see Figure 13 A. Szabo *et al.* have reported that when a solution containing KCl and IgG is dried out on a surface, the presence of IgG modifies the KCl crystal growth and IgG covers the surface of the crystals formed, see Figure 13 B[71]. Despite the fact that in these experiments proteins are adsorbed on the stamp surface prior to drying, it would seem that proteins are removed from

the stamp surface to cover the surface of the formed crystals. The homogeneity of the protein layer is therefore lost. Furthermore, the salt crystals formed were seen to prevent conformal contact between stamp and surface during transferral of proteins. Careful washing of stamps in water should therefore always be performed prior to drying.

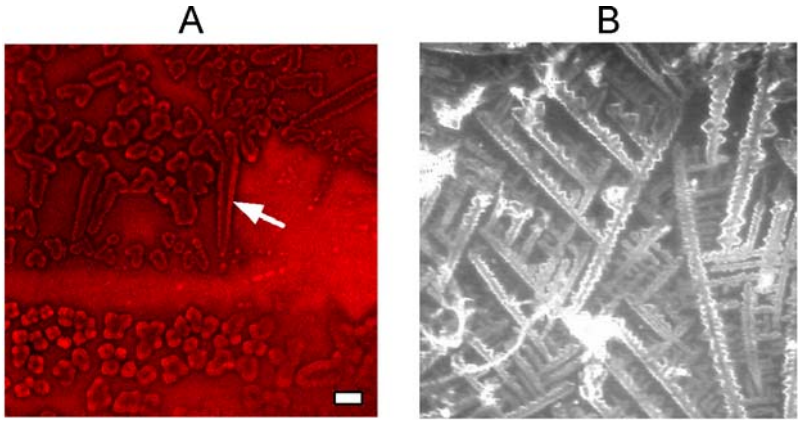


Figure 13 Fluorescence microscopy images of (A) IgG-Alexa inked stamp after drying from a salt containing solution. Scale bar is 10μm. (B) KCl crystals formed by drying out a drop containing KCl and fluorescence labelled IgG. The bright contour of the crystals is due to the labelled IgG covering the surface[71]. Image size is 135x135 μm^2. (Reprinted with permission from APS.)

3.5 Protein redistribution at water stamp contact lines

When examining inked stamps in fluorescence microscopy, inhomogeneously coated areas that primarily had rounded shapes were very frequently observed. These structures are due to water droplets redistributing proteins as they move across or dry out on the surface. Several different approaches were tried in order to develop a method for controlled removal of water from the protein-coated stamps. One approach was to retract stamps from the washing water using a langmuir trough. Water must undoubtedly move across the stamp surface also when it is dried using an argon flow. Moreover, water does not redistribute proteins in an inhomogeneous fashion under all conditions, as not all areas are inhomogeneously coated. We therefore hypothesized that if stamps were pulled through the water air interface slowly enough to avoid leaving drops of water on the surface this might produce a homogeneous coating.

Stamps, containing 380 μm wide raised plateaus separated by 390 μm wide channels (app. 0.5 cm^2), were mounted in the langmuir trough and immersed in 1 ml of a 50 μg/ml solution of IgG-Alexa. The stamps were oriented such that channels were perpendicular to the water surface. After 10 min incubation, stamps were rinsed by adding copious amounts of Millipore® water to the container in which the stamp was immersed. Subsequently stamps were retracted from the water, in the direction of the ridges, at different velocities. For reference

another set of stamps were immersed, incubated and rinsed by addition of water as just described, but these stamps were dried using a gentle flow of argon. Fluorescence microscopy revealed significant differences in the coating achieved using the two methods. Figure 14 shows a stamp retracted from solution at 0.1 mm/s and a stamp dried with argon. Stamps dried using argon show a comparable coating of ridges and channels while on stamps retracted slowly from the water, proteins have been partially removed from the raised areas of the stamp surface.

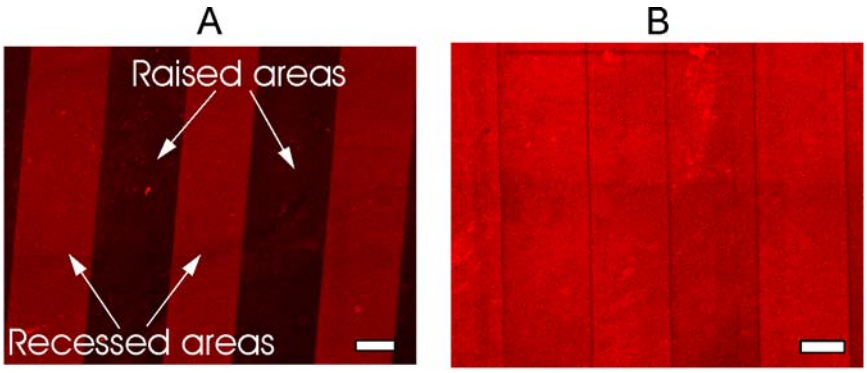


Figure 14 Fluorescence microscopy images of (A) stamp dried by use of langmuir trough. (B) Stamp dried under a flow of argon. Scale bars are 200 μm.

It is thus clear that this method is not suitable for coating structured stamps. The distinct difference in protein coating of the ridges and channels indicates that the degree of redistribution of protein at the water stamp contact line is highly dependent on the shape of the meniscus moving across the surface.

3.6 Spin drying

Spin coating is the most widespread technique for applying thin uniform coatings on flat surfaces and it is used routinely to deposit e.g. photoresists and insulating layers for fabrication of microcircuits, protective coatings and antireflection coatings. Roughly the spin coating process can be separated in four process parts, as shown in Figure 15: (A) dispensing of the material to be distributed on the substrate; (B) acceleration of the substrate to a final rotation speed; (C) spinning at constant rate while the material layer on the substrate is thinning due to radial forces causing material to be ejected at the edges of the substrate and finally (D) spinning at constant rate while excess solvent evaporates[72].

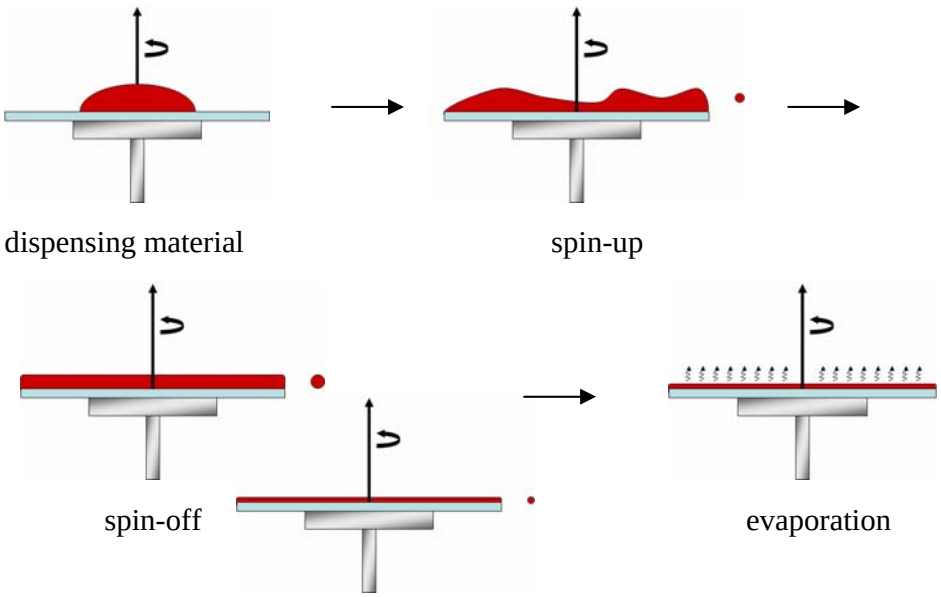


Figure 15 Schematic figure illustrating the four process parts in spin coating.

In the standard applications of spin coating, the material to be deposited on the substrate is immobilized on the substrate surface as a consequence of the spin coating process. First it is distributed evenly on the surface through the rotation of the substrate and subsequently it is immobilized as the solvent evaporates. Due to the spontaneous adsorption of proteins on a hydrophobic stamp surface, this would not be the case if one attempted to spin coat protein solutions on stamps. However, if proteins were allowed to adsorb on the stamp surface prior to spinning, this partial immobilization could prevent redistribution of proteins during the spin off of water. Furthermore, the gradual thinning and evaporation of the water-film could provide an excellent method for removing water in a homogeneous manner over the entire stamp surface thus reducing the redistribution of proteins due to water menisci.

3.6.1 Spin drying flat stamps

As spin coating is normally utilized on flat substrates, a natural starting point was to examine whether homogeneous coating of flat stamps could be achieved by use of spin-drying. Flat stamps (app.1 cm^2) were placed on a microscope slide so that conformal contact was achieved. 150 µl of a 50 µg/ml solution of IgG-Alexa was placed on the stamp surface. After 10 min incubation the glass slide and stamp were mounted in a spin coater (Laurell WS-400A-6NPP-lite) and 2 ml of Millipore® water was added to remove protein solution and loosely bound proteins. Immediately after adding water, the stamps were dried by spinning 10-30 s at 2500 rpm with an acceleration time below one second. Remaining drops along the edges of the stamp were gently removed with a tissue. After this, stamps were printed on a cleaned cover glass. The central part of Figure 16 shows a low-resolution (5x objective) image of such a micro contact printed glass surface.

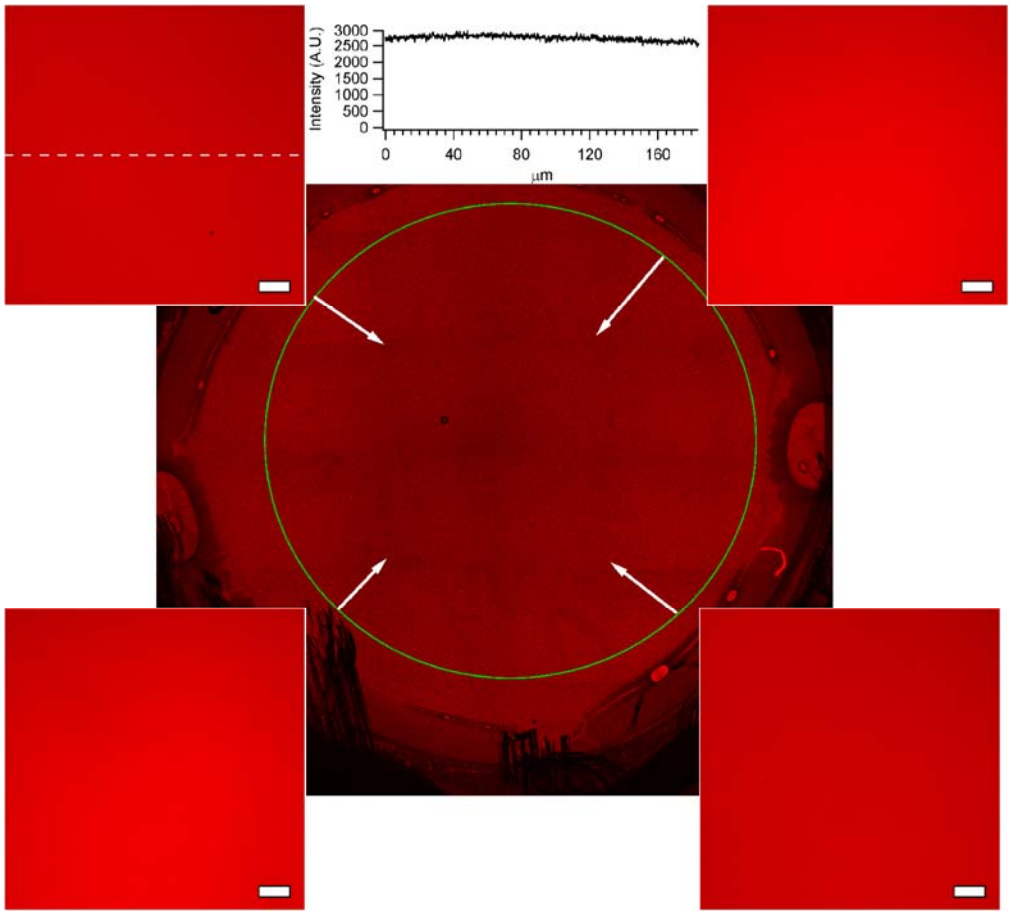


Figure 16 Fluorescence microscopy images of a glass surface micro contact printed with a flat stamp dried by spinning.

The image is a tiled fluorescence image recorded using the automated stage of the microscope. The drop of solution applied to the stamp did not cover the entire stamp surface and therefore only the central region indicated by the green circle is considered. Each separate image in the tile can easily be distinguished due to a microscope-related intensity variation across each image field. However, the image clearly demonstrates an overall homogeneous coating with no noticeable intensity variations within the central area apart from the dark spot in the centre. Fluorescence and bright-field images recorded of the stamp after printing reveal that the lack of transfer in this area was due to a particle on the stamp preventing contact with the substrate. In this not transferred area the average intensity is 231 A.U. ± 16 (SD) whereas in the remaining central part of the stamp the average intensity is 15 A.U. ± 2 (SD) which demonstrates efficient transfer to the substrate. High-resolution images of the printed substrate (50x objective) were recorded to obtain information on the homogeneity of the coating and to determine if the coating contained defects that could not be detected at low resolution. In Figure 16 four such images and a line profile along the dashed white line in the upper left image are shown. The presented images were recorded in the positions indicated by the white arrows in the centre image. It is

clear that, also at high resolution the coating shows very few defects and all high-resolution images recorded on samples dried by spinning show standard deviations of 4% to 7%. This confirms that removal of water by spinning provides an excellent method for drying flat stamps avoiding water-induced inhomogeneities.

As stated earlier Michel *et al.* have demonstrated high-resolution micro contact printing using flat stamps and subtractive inking[41]. Therefore, the use of homogeneously coated flat stamps offers the possibility of producing features with sizes varying from the size of the coated area (here 9 mm) down to app. 100 nm. For production purposes the process of subtractive inking possesses one disadvantage. The structured substrate utilized in the subtractive inking process must be cleaned between each stamping cycle, thus introducing an additional process step. We therefore investigated whether the method of spin-drying might be applicable to structured stamps.

3.6.2 Spin drying structured stamps - large structures

As the homogeneous distribution of material (and solvent) in spin coating is related to the flatness of the substrate we did not expect to be able to achieve comparably homogeneous coatings when applying the method to structured stamps. This was certainly true when using stamps containing large structures. Figure 17 shows a cover glass printed using a stamp containing a meander pattern of 380 μm wide ridges. Stamps were inked and dried following the same procedure as described for flat stamps.

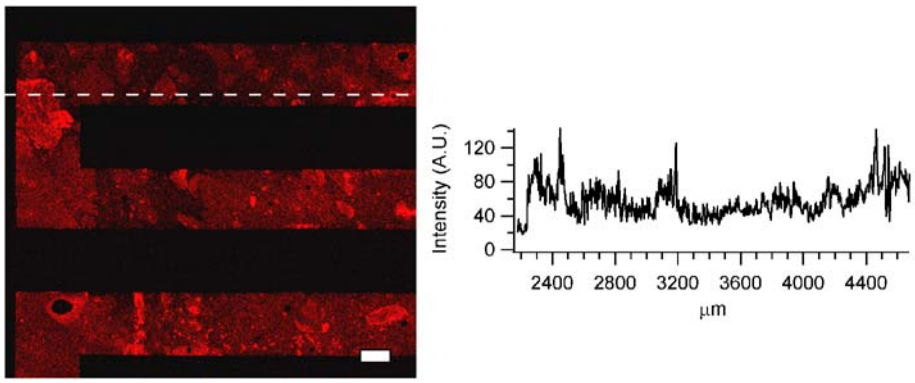
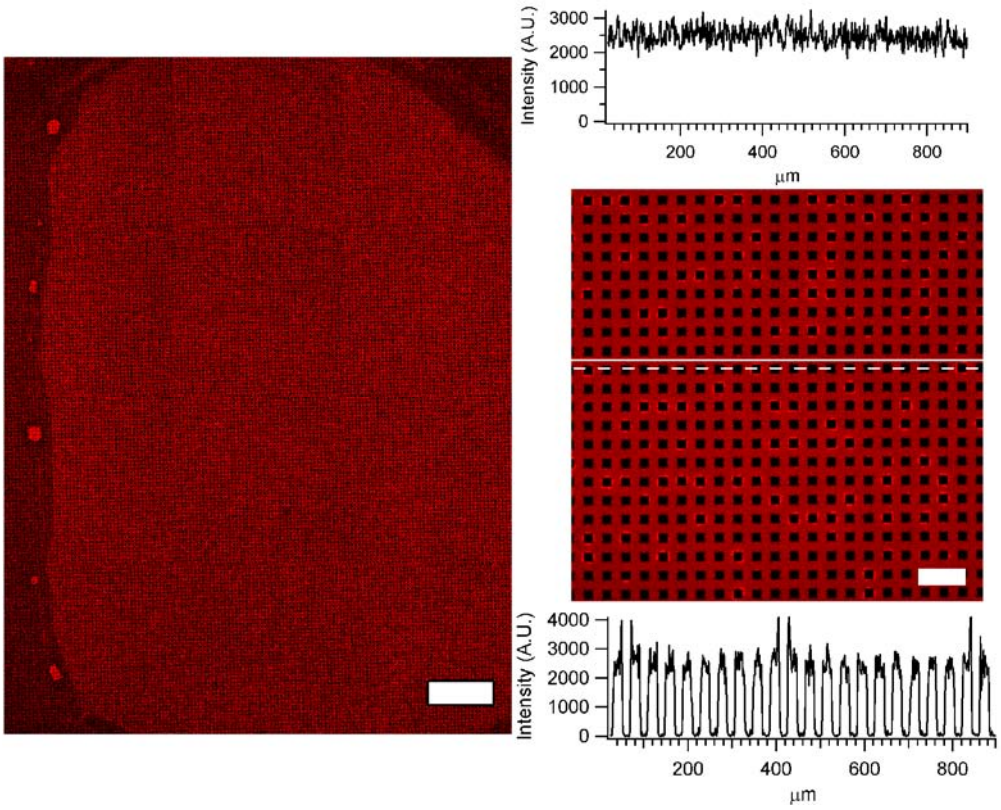


Figure 17 Fluorescence microscopy image of a glass surface printed with a spin dried stamp. The line profile shows intensities along the path indicated by the dashed white line. Scale bar 100 μm.

The average intensity in the patterned area is 71 A.U. ± 22 (SD) with a background intensity of 27 A.U. ± 7 (SD). This and the inserted line profile clearly demonstrate a very inhomogeneous coating density. Fluorescence microscopy images of the stamp after it had been used for printing showed no retained protein. Therefore, the inhomogeneity of the transferred pattern could not be ascribed to poor transfer, thus ruling out the possibility of utilizing spin-drying when coating stamps with structures of this size.

3.6.3 Spin drying structured stamps - small structures

A markedly better homogeneity was achieved when we employed stamps containing a raised grid consisting of 20 μm wide lines separated by 20 μm in each lateral direction. In these experiments fluorescence labelled avidin, (Avidin Texas Red conjugate, Sigma-Aldrich) (avidin-TR) was used instead of IgG-Alexa. Stamps were coated by placing a 200 μl drop of a 100 μg/ml solution of avidin-TR on top of the stamp, and spreading it out with the pipette tip, carefully avoiding to touch the stamp. After a 10 min incubation the glass slide with the stamp was mounted in the spin coater, washed and dried as described previously.



A B

Figure 18 Stamp coated with avidin and dried by spinning. The line profiles show intensities along the paths indicated by the full white line (upper profile) and the dashed white line (lower profile). Scale bars (A) 1 mm (B) 100 μm.

From the tiled low-resolution (5x objective) fluorescence image in Figure 18 it is clear that the central part of the coated stamp shows no large-area intensity variations. High-resolution images (20x objective) reveal that along the edges of a large part of the wells, there are areas of between 1 and 5 μm width showing app 50 % increased intensity. However the remaining areas are far more homogeneously coated showing an average intensity 2425 ± 243 (SD) with a background of 53 ± 55 (SD). Examining the stamp using confocal fluorescence microscopy reveals that within the central area none of the wells are protein coated and thus they have not been filled with solution during the inking. A few larger high intensity areas can be observed along the edges of the central

homogeneously coated area. This is the area where drops of solution often remained after spinning. In these areas and only here, wells are coated. It seems likely that the large improvement in homogeneity, as compared to the stamps containing 380 μm structures, is a result of the confinement of the inking solution to the raised areas of the stamp. We suggest that the explanation for the good homogeneity and the high intensity areas surrounding wells is the following. During the early stages of spinning, when a thick layer of water is present on the surface, the flow across the stamp is similar to the flow over a flat surface. However, as the thickness of the water layer is reduced it is no longer energetically favourable to maintain a water layer spanning the gaps across wells and as these burst, drops are trapped along the edges of the wells where they remain and dry out.

In summary the presented results show that spin-drying is not immediately applicable to structured stamps. However, by spin-drying flat stamps it is possible to achieve large (>1cm^2) homogeneously coated areas. This may be utilized in subtractive inking through which patterns with feature sizes down to 100 nm can be produced.

3.7 Protein transfer in printing

3.7.1 Relevant parameters

When a homogeneous coating of stamps has been achieved, proteins must be transferred efficiently to the end substrate. Chen and coworkers carried out a careful study of the dependence of transfer efficiency on substrate and stamp wettability[32]. They micro contact printed proteins on mixed self-assembled monolayers (SAMS) presenting increasing amounts of polar functionalities and found that efficient transfer was closely related to the presented mole fraction of polar functionalities. No protein was transferred at low molar fractions and as the fraction was increased the switch between no transfer and complete transfer occurred within a narrow threshold range. The point of switching occurred at molar fractions that produced virtually identical advancing contact angles for SAMS presenting -OH, -COOH and $–(OCH_2CH_2)_6OH$ functionalities. Furthermore, they found that when the hydrophobicity of the stamp was increased, it was possible to transfer proteins to surfaces that could not be patterned using unmodified stamps. They therefore concluded that, in order to transfer proteins efficiently, there must be a certain difference in the wettability of stamp and substrate. Thus, utilizing hydrophobic stamps should produce effective transfer on a broader range of substrates than utilizing hydrophilic stamps.

This may, however, not be true for all proteins. Hydrophobic stamps have been reported to exhibit poor transfer as compared to hydrophilic stamps when utilized for printing cytochrome-c on indium tin oxide (ITO) coated surfaces. Runge *et al.*[29] performed Attenuated Total internal Reflection (ATR) spectroscopy studies of cytochrome-c adsorption on hydrophilic and hydrophobic stamps. They found

that similar amounts of protein were adsorbed on hydrophobic and hydrophilic stamps. However, x-ray photoelectron spectroscopy studies of printed ITO surfaces revealed much lower protein density on surfaces printed using hydrophobic stamps than on surfaces printed using hydrophilic stamps. It is therefore possible that other parameters than wettability influence the transfer.

3.7.2 Protein transfer - glass and silicon

Successful micro contact printing of proteins on glass and silicon surfaces has been reported by numerous groups[28,73-75]. We carried out several series of printing-experiments using glass and silicon substrates. In all the performed experiments we found that, as described in literature, proteins transfer readily from the stamp surface onto these substrates. As already illustrated by the results presented in Figure 16 transfer occurs homogeneously over large areas. Figure 19 shows an overview fluorescence image and a high-resolution image of a silicon surface micro contact printed with a stamp containing 20x20 μm square raised areas inked by placing a 150 μl drop of solution containing 50μg/ml IgG-Alexa on the stamp surface. After incubation and washing, the stamp was dried in a flow of argon and placed on the cleaned silicon surface.

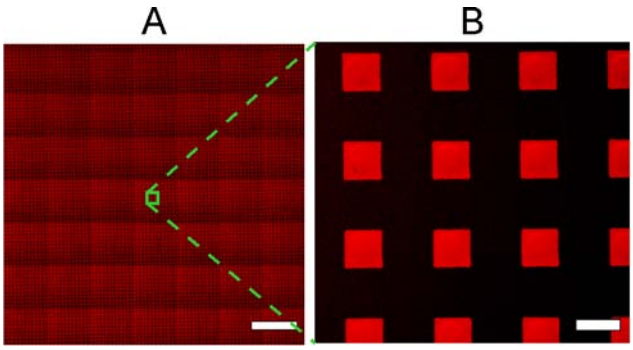


Figure 19 Fluorescence microscopy images of IgG-Alexa micro contact printed on a silicon surface. Left image is a tiled image recorded using the automated stage of the microscope. Scale bars (A) 500 μm (B) 20μm.

3.7.3 Protein transfer - polystyrene and polymethylmethacrylate

After having established that we could transfer proteins to glass and silicon, we performed a series of printings on unmodified polystyrene (PS) and polymethylmethacrylate (PMMA) surfaces. Transfer to PS and PMMA surfaces using unmodified stamps has been reported previously[27]. Stamps containing 40x40 μm^2 or 20x20 μm^2 square raised areas were inked and printed as described in the experiment above. Fluorescence microscopy images of the printed surfaces and of the stamps after printing reveal highly irregular transfer (see Figure 20).

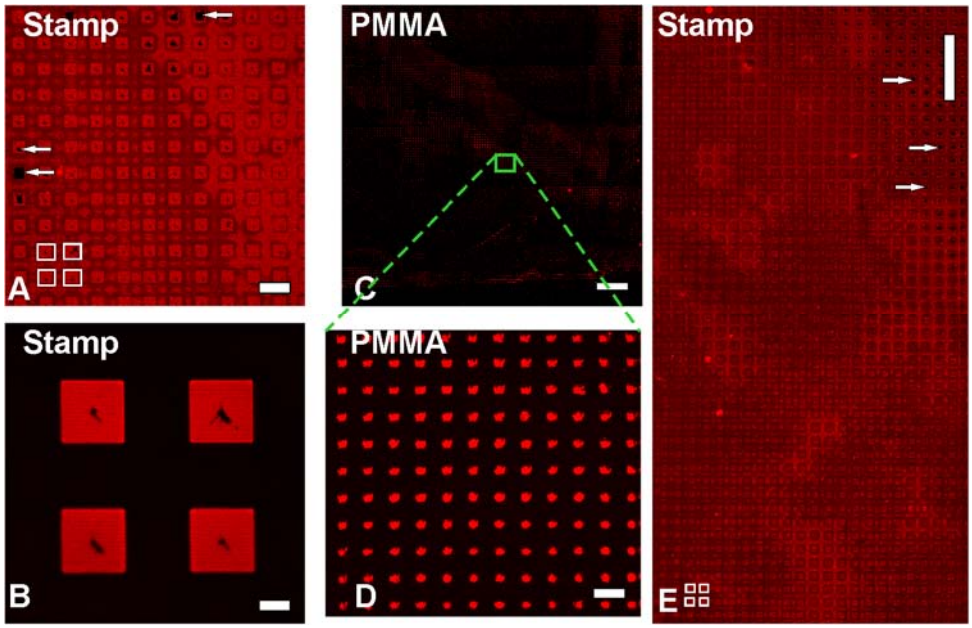


Figure 20 Fluorescence microscopy images of IgG-Alexa on: (A-B) Stamp after printing on a PS surface. (C-D) Printed PMMA surface (E) Stamp after printing on a PMMA surface. White arrows have been inserted to mark examples of areas on the stamps where partial transfer has occurred. White squares on images of printed stamps indicate the size of the raised areas. Scale bars (A) 100 μm (B) 20 μm (C) 500 μm (D) 50 μm (E) 200 μm.

On both PS and PMMA surfaces we located areas where transfer had occurred. However, transfer of an entire square patch was rare and as can be seen in Figure 20 C there was no observable transfer in many areas. It has been reported that transfer efficiency decreases when stamps are not placed on the substrate surface within 1 min (at 55 % ambient humidity)[27]. To ensure that the poor transfer was not caused by too long time used for drying the stamps, we lowered the drying time in argon to a minimum, still ensuring that no water was left in the channels on the stamp. This did, however, not improve transfer. We were thus not able to achieve reproducible, uniform transfer of proteins onto these surfaces and did not pursue this strategy any further.

3.7.4 Protein transfer - plasma treated polystyrene

Tan *et al*. reported improved transfer when substrate wettability was increased[32] and successful protein transfer to Tissue Culture Polystyrene (TCPS) substrates has been reported[32,75]. TCPS is produced by exposing polystyrene parts to an atmospheric plasma (corona) treatment rendering the surfaces highly hydrophilic. We performed a series of printing experiments using polystyrene surfaces that had been exposed to an air-plasma. The untreated polystyrene surfaces were produced in-house on a VC 80/25 injection moulding machine (Engel, Schwertberg, Austria) by Henrik Pranov using Polystyrol 158K (BASF, Ludwigshafen, Germany). Plasma treatment was performed in a home-built plasma chamber with parallel plate electrodes of 100 mm diameter separated by

70 mm using 30 W and 13.56 MHz rf excitation at a pressure of 0.5 mbar. Surfaces were exposed to the plasma for 5 to 30 s and were subsequently stored in water to ensure that no radicals remained on the surface when they were used for printing. Atomic Force Microscopy (AFM) images recorded of replicas prior to and after plasma treatment demonstrated that the topography of the surfaces was not altered in the process, see Figure 21. Unless otherwise noted all AFM images were obtained using a Dimension 3000, Veeco Metrology Santa Barbara, CA and all measurements were performed in tapping mode using PointProbe cantilevers (NanoSensors, Neuchatel, Switzerland)..

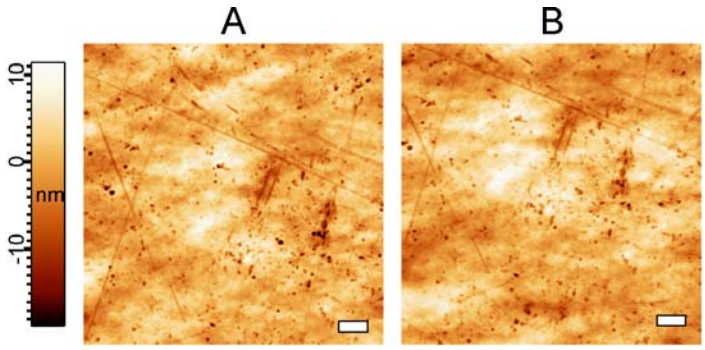


Figure 21 AFM images of polystyrene surface before (A) and after (B) a 30 s exposure to an air-plasma. Scale bars 1 μm.

Plasma treated polystyrene (PSox) surfaces were micro contact printed using stamps containing 40x40 μm square raised areas inked by placing a 200 μl drop of solution containing 25 μg/ml IgG-Alexa on the stamp surface. After incubation and washing the stamps were dried in a flow of argon and immediately after this placed on the dry PSox surfaces.

Fluorescence microscopy images of the printed surfaces show excellent transfer over large areas as seen when printing on glass and silicon surfaces, see Figure 22 A-C. Images of stamps after printing show an average intensity of 281 A.U. ± 24 (SD) in raised areas demonstrating efficient and uniform transfer from stamps to PSox. There was no detectable difference in transfer between surfaces plasma treated 5 and 30 s. Transfer onto plasma treated surfaces that had not been stored in water after plasma treatment produced equivalent results.

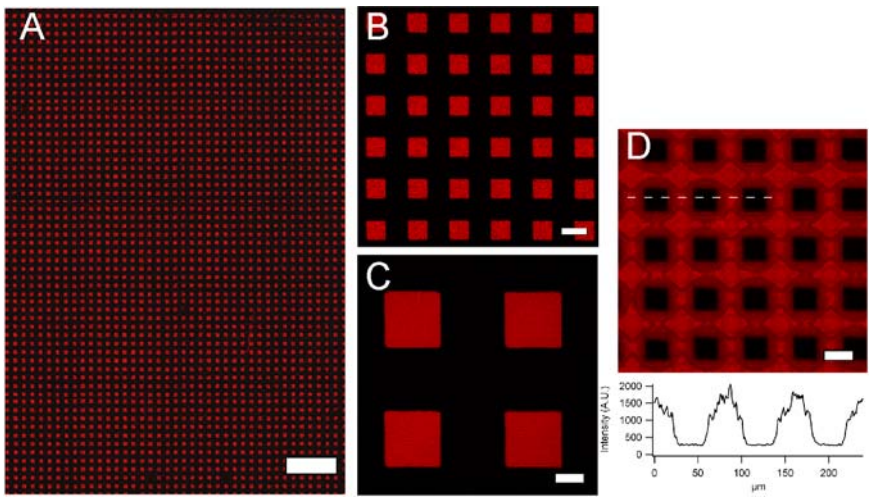


Figure 22 Fluorescence microscopy images of (A-C) IgG micro contact printed on air-plasma treated polystyrene. (D) Stamp after printing. Scale bars (A) 500 µm (B) 50 µm (C) 20µm (D) 50 µm.

In conclusion, we find that proteins transfer readily onto hydrophilic surfaces such as glass, silicon and air-plasma treated PS and on these substrates transfer is uniform over large areas, 1x1 cm^2 being the largest tested. On unmodified PS and PMMA we were not able to achieve uniform transfer and therefore conclude that micro contact printing using unmodified stamps is not a viable method for patterning these polymer surfaces.

3.8 Stability of printed patterns in medium

Proteins patterned by micro contact printing are generally considered to be immobilized on the substrate[26]. To ensure that this was also the case for our samples a stability test was performed. The extracellular matrix protein fibronectin is commonly used to induce adherence of cells in culture and could be a good candidate for use in production of cellular arrays. We therefore chose to test the stability of micro contact printed patterns of fibronectin when stored in serum-supplemented cell culture medium at 37°C. Stamps containing a meander pattern of 400 µm wide raised areas were coated by placing a 200 µl drop of solution containing 50 µg/ml fibronectin from human plasma (Sigma-Aldrich). After 10 min incubation, stamps were rinsed in water and dried in a flow of argon. Immediately after this, they were placed on polystyrene surfaces that had been given a 5 s air plasma treatment at 0.5 mbar, 30W and had subsequently been stored in water. This process ensured that no radicals remained on the surface when they were used for printing. As the fibronectin was not fluorescence labelled, samples were immunolabelled prior to storage in medium. Unspecific adsorption of antibodies during the labelling was minimized by blocking uncoated surface areas through a 15 min incubation with a 50 µg/ml solution of Human Serum Albumin (HSA). Replicas were immunolabelled by incubating: first with a primary antibody (monoclonal anti-fibronectin antibody

produced in mouse Sigma-Aldrich); second incubating with secondary antibody IgG-Alexa. Between each incubation step replicas were rinsed in copious amounts of PBS. Samples were then rinsed in water, dried, visualized in fluorescence microscopy and finally placed in 5 ml serum supplemented cell culture medium at 37^{o}C where they remained for 3 days. Before visualizing samples after wet-storage they were rinsed in water and dried to avoid salt crystals on the surfaces.

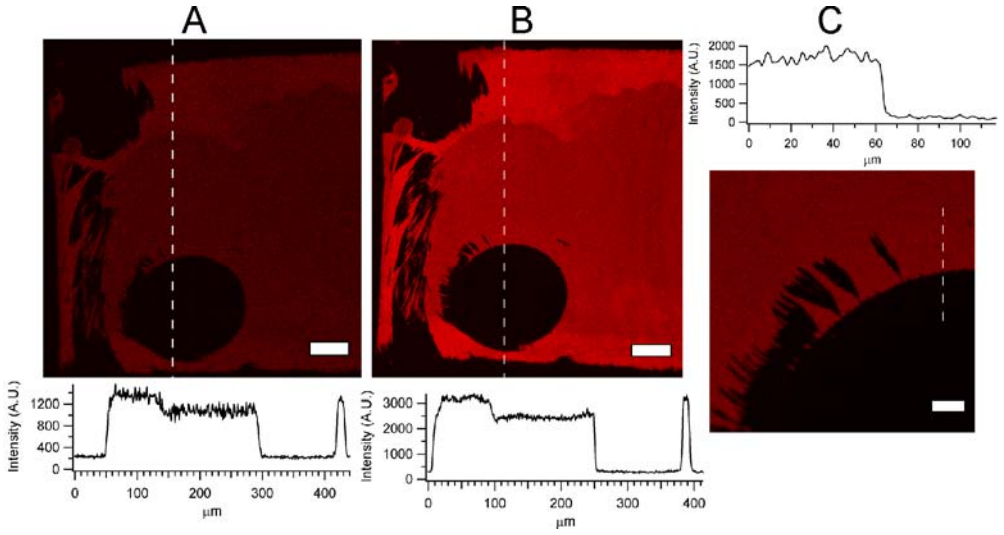


Figure 23 Fluorescence microscopy images of plasma treated polystyrene surface micro contact printed with fibronectin. (A) Prior to storage in medium. (B) After storage in medium. (C) After storage in medium. Scale bars (A-B) 50 μm. (C) 10 μm.

The fluorescence images (A) and (B) presented in Figure 23 show an area of imperfect transfer before and after wet-storage. These images were recorded using the same microscope settings apart from using a lower gain when recording image (B). The inserted line profiles for these two images show the average intensity in a 20 μm wide band along the indicated white dashed lines. The average intensity of the centre area has increased from 1065 A.U. ± 296 (SD) to 2419 A.U. ± 306 (SD) despite the use of a lower gain. At present we do not have an explanation for this increase in intensity that was present on all prepared samples. It is, however, likely to be due to a variation in the intensity of the laser used to excite the fluorophores. Due to this it is not possible to compare average values from images recorded before and after storage in media. However, the relative intensities of the different areas have not been altered by the storage in medium indicating that if material desorbs from the surface it does so evenly over the surface. An estimate of the amount of protein adsorbed in the background area after wet-storage was calculated by assuming that the intensity in the protein coated area in image (C) corresponds to a full monolayer. This is likely to be an overestimate since micro contact printing transfers mono- or submonolayers of protein and areas of higher intensity are present on the sample. We further assume a background intensity of zero, which surely is an underestimate. Using these assumptions the average intensity in the unpatterned

areas corresponds to 6 % of a monolayer. We therefore consider the micro contact printed proteins immobilized on the surface which confirms the findings of other groups.

3.9 Overlap micro contact printing

For many applications the patterning of several different proteins on a surface is a necessary prerequisite for device production. One strategy to achieve this is based on consecutive overlaid printings on the same substrate, i.e. overlay-printing. This method was introduced by Bernard *et al.*[27] and was later employed by Inerowicz *et al.* to fabricate protein arrays capable of detecting up to three different ligands[76]. The method is based on two assumptions:

- On a surface patterned with a mono or sub-monolayer of proteins by micro contact printing, a subsequent printing will only transfer protein to bare surfaces areas.
- Micro contact printing on a surface patterned by micro contact printing does not remove protein from the surface.

Overlay-printing is an appealing strategy for patterning multiple proteins because it provides a way to pattern several different proteins in separate areas on a surface. Furthermore, it offers the opportunity to form areas containing more than one type of protein through transfer of sub-monolayers and filling of the remaining free surface areas in subsequent printings. However, Inerowicz *et al.*[76] reported that AFM studies of the patterned areas suggested that two overlapping layers of protein were present in the overlay-printed regions.

Determining whether this is the case is important, as overlapping layers would most likely cause steric hindrance of part of the proteins deposited in the first printing. We investigated this by performing overlay-printing on glass surfaces and examining the resulting protein patterns in AFM. We chose to print on glass as these surfaces are more planar than polymer substrates, which facilitates evaluation of the protein layer thickness. To facilitate verification of transfer we used fluorescence labelled fibrinogen (Alexa fluor 488 fibrinogen conjugate from Molecular Probes)(Fibrinogen-488) and fluorescence labelled fibronectin (fibronectin-546). Fibronectin was labelled using an Alexa Fluor 546 Protein Labelling Kit from Molecular Probes and following the manufacturer recommendations. After labelling, the protein concentration was determined by use of a BCA™ protein assay from Pierce. The concentration determination was kindly performed by Dr. Danielle Keller. The stamps used contained 20 x 20 μm^2 square raised areas separated by 20 μm in both lateral directions. Stamps (app. 0.5 cm^2) were coated by applying a 200 μl drop of a 1μg/ml solution of fibronectin-546 in DPBS on the stamp surface. After 5 min incubation, the stamp was washed in Millipore® water, dried in a stream of air and placed on the coverglass immediately after drying. Subsequently the same procedure was performed using a 30 μg/ml solution of fibrinogen-488.

Figure 24 presents fluorescence microscopy images of an overlay printed surface. In the upper images fluorescence from both fibronectin-546 and fibrinogen-488 is recorded. The lower images show fluorescence only from fibrinogen-488 in the same areas. The transfer of fibrinogen is seen to be excellent in all bare glass areas, but in the fibronectin coated areas transfer varies markedly. In some fibronectin areas there is no detectable transfer and in all the areas where transfer occurred, less protein was transferred to fibronectin-coated areas than to the immediately surrounding bare glass. According to the assumptions, this should indicate a high density of fibronectin in areas where no transfer occurs and a lower density in the remaining areas. However from the intensity of fibronectin-546 such a correlation is not obvious.

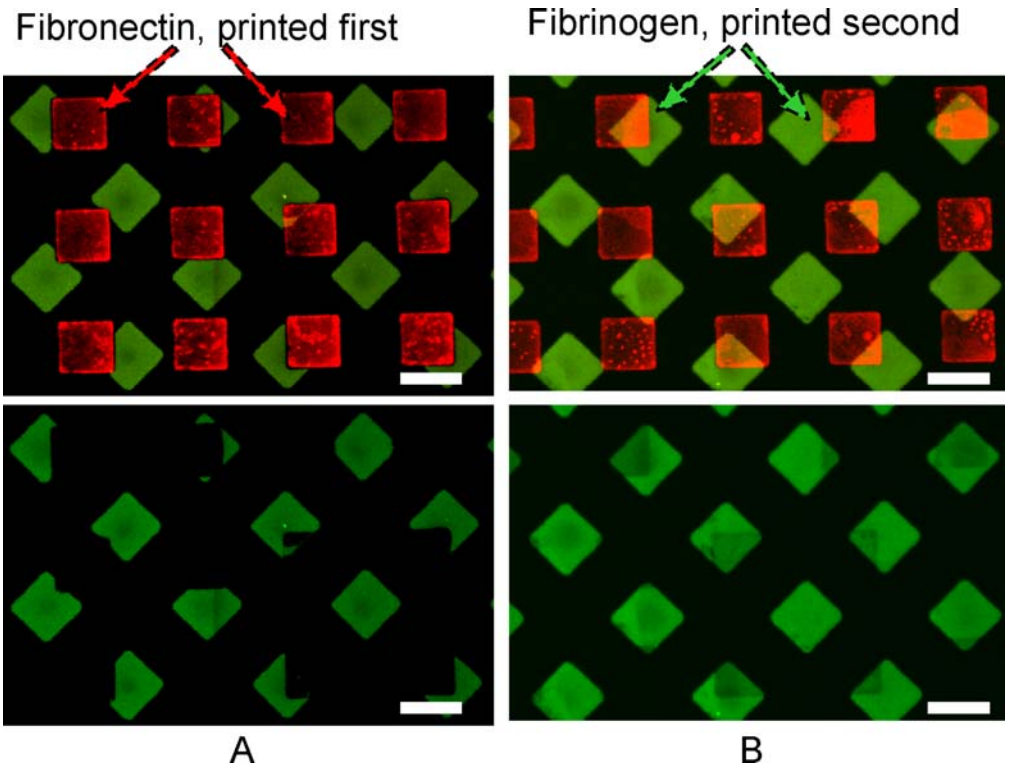


Figure 24 Fluorescence microscopy images of glass surface patterned with fibronectin (red) and fibrinogen (green) by overlay printing. The upper and lower images are recorded in the same area and both sets of images are recorded on the same samples. Scale bars are 20 μm.

AFM measurements of areas where transfer has occurred show a thicker protein layer in areas where both fibronectin and fibrinogen is present, see Figure 25.

Area	2 Fibronectin	3 Fibronectin and fibrinogen	4 Fibrinogen
Height [nm]	3.143	7.122	3.628

Table 1 Average height of the coated numbered areas in Figure 25 after subtraction of background.

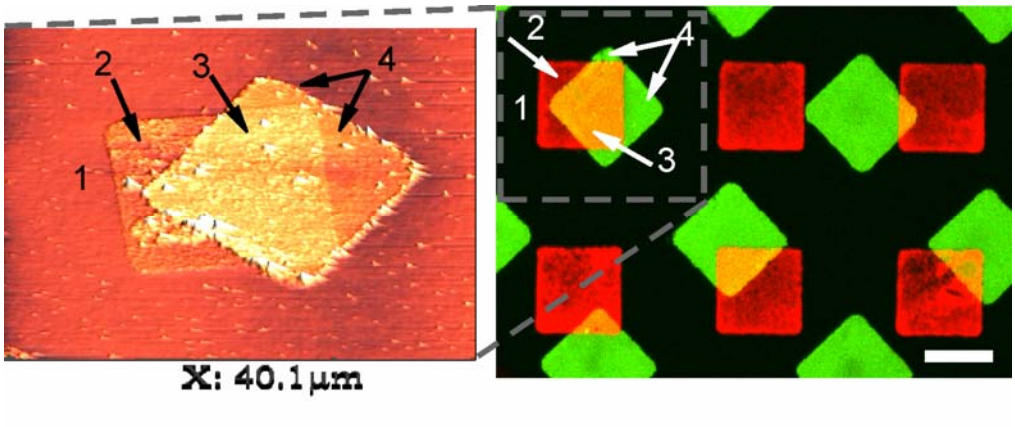


Figure 25 Atomic Force Microscopy image and fluorescence microscopy image of overlay printed area. The numbers in the two images indicate the areas used when calculating the average heights in the AFM image. Scale bar 10 µm.

The average height of the (1) background (2) fibronectin coated area (3) fibronectin and fibrinogen coated areas and (4) fibrinogen coated areas were determined and the background height subtracted. This reveals that fibrinogen is deposited in a layer on top of the previously deposited fibronectin see Table 1. The imaged surfaces have not been immersed in solution following transfer and it is thus possible that fibrinogen transferred on top of fibronectin could be rinsed off. However, Inerowicz *et al.*[76] imaged overlay printed surfaces in wet condition and still observed an increased height in the overlay printed region. It therefore does not seem likely that, with the experimental conditions reported, overlay printing can produce surfaces with well-controlled intermixed protein densities in the overlay printed regions.

3.10 Protein networks

In 2004 Sgarbi *et al*. published work on the micro contact printing of laminin[77], a network forming extracellular matrix glycoprotein that is present in the basal lamina (see glossary). They reported that AFM studies revealed formation of physiological network structures of laminin-1 when inking-solutions containing 10 µg/ml were employed. When the protein concentration was increased, homogeneous transferred layers with no detectable network structure resulted. This was ascribed to the formation of 3D network structures in solution that collapse on the stamp surface during drying, thus causing a dense structure in which the network cannot be detected. Yurchenco *et al.*[78] have shown that the

presence of Ca^{2+} is necessary for laminin-1 network formation. As calcium-free solutions were utilized, the formation of network structures was ascribed to a possible Ca^{2+} contamination of the PBS solution used in the experiments.

During our AFM examinations of micro contact printed proteins we observed network structures very similar to the structures reported by Sgarbi *et al*.

In Figure 26 is shown a fluorescence image of IgG-Alex micro contact printed on a silicon surface by use of stamps containing 40 x 40 μm^2 raised square structures. Stamps were inked using a 50 μg/ml solution of IgG-Alexa and were incubated 10 min before rinsing in water and drying with argon. Judging from the fluorescence images the transferred square areas appear homogeneously coated but AFM measurements reveal clear network structures in the printed protein layer, see Figure 26.

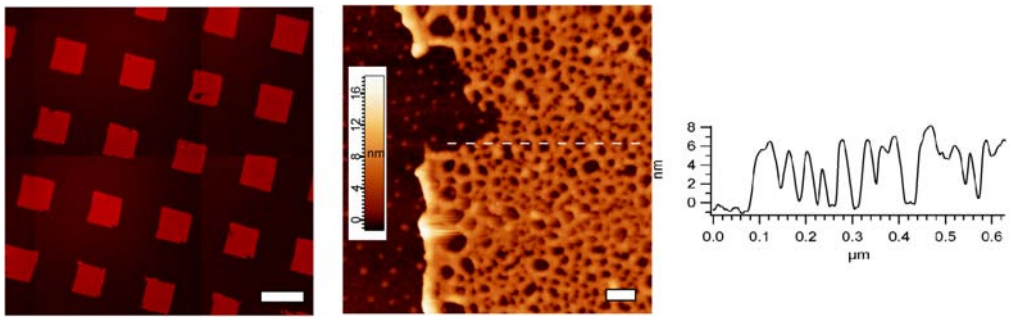


Figure 26 Fluorescence and atomic force microscopy images of a silicon surface patterned with IgG-Alexa by micro contact printing. The inserted line profile shows the height profile along the dashed white line. Scale bars (A) 50 μm (B) 100 nm

However, IgG is not a network-forming protein. We therefore expect that it is another mechanism than network formation in solution that is the cause of the formed structures. Network patterns were observed for IgG-Alexa, Collagen IV and fibronectin and were observed both when printing on silicon and on air-plasma treated polymer surfaces. The concentrations used when inking stamps were 50 μg/ml for IgG-Alexa and collagen and 2 μg/ml for fibronectin in Figure 27. The formation of network structures at 50 μg/ml concentration was only observed a few times. Using lower concentration these structures were observed far more frequently.

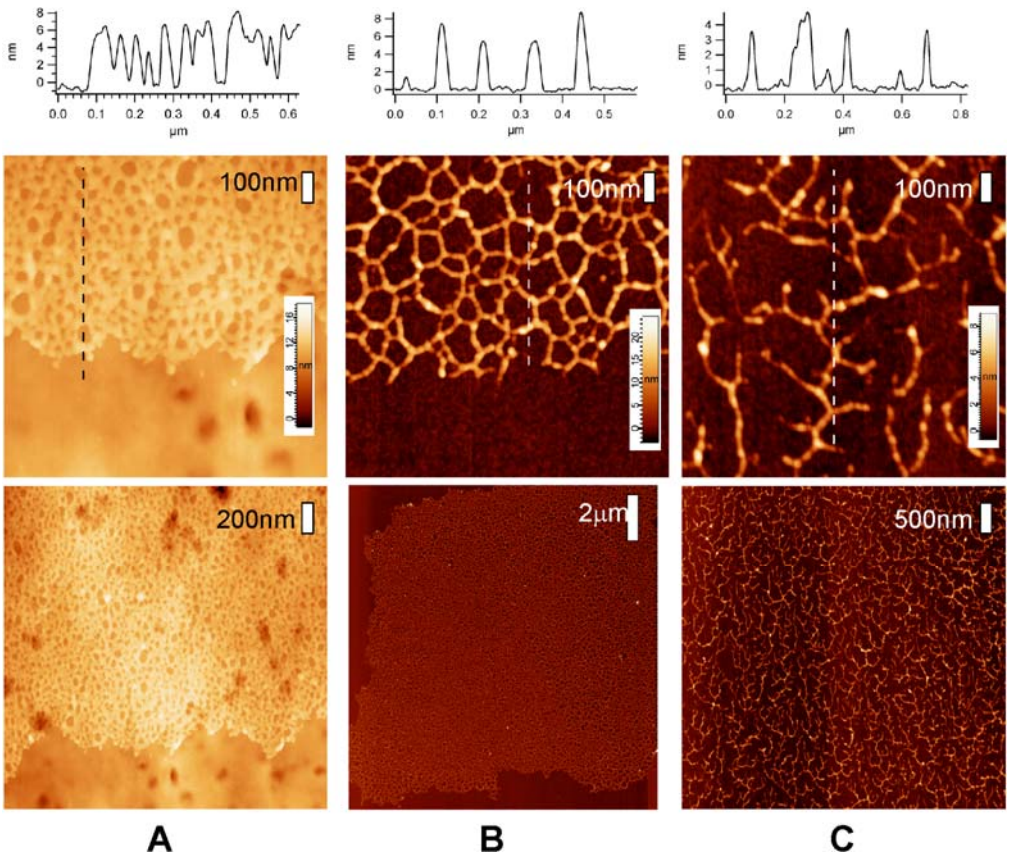


Figure 27 (A) IgG-Alexa micro contact printed on air-plasma treated polystyrene. (B) Fibronectin micro contact printed on silicon. (C) Collagen IV micro contact printed on silicon.

Using fibronectin we performed a series of printings varying the protein concentration in the inking solution. Stamps with 40 x 40 μm^2 square raised areas were inked by placing a 200 µl drop of solution containing fibronectin on the stamps surface. After a 10 min incubation the stamps were rinsed in PBS and Millipore® water, dried in a stream of argon and printed on a cleaned silicon surface. Samples were imaged in AFM.

The structure of the transferred protein layer varied within different squares on the same sample and we often observed a denser network in the centre of each square area than close to the edges of the square. However, the general trend was formation of an increasingly dense network as the protein concentration in the inking solution was increased (see Figure 28 A). At concentrations higher than 5 µg/ml we observed either dense protein layers or dense protein layers interrupted by holes of various sizes.

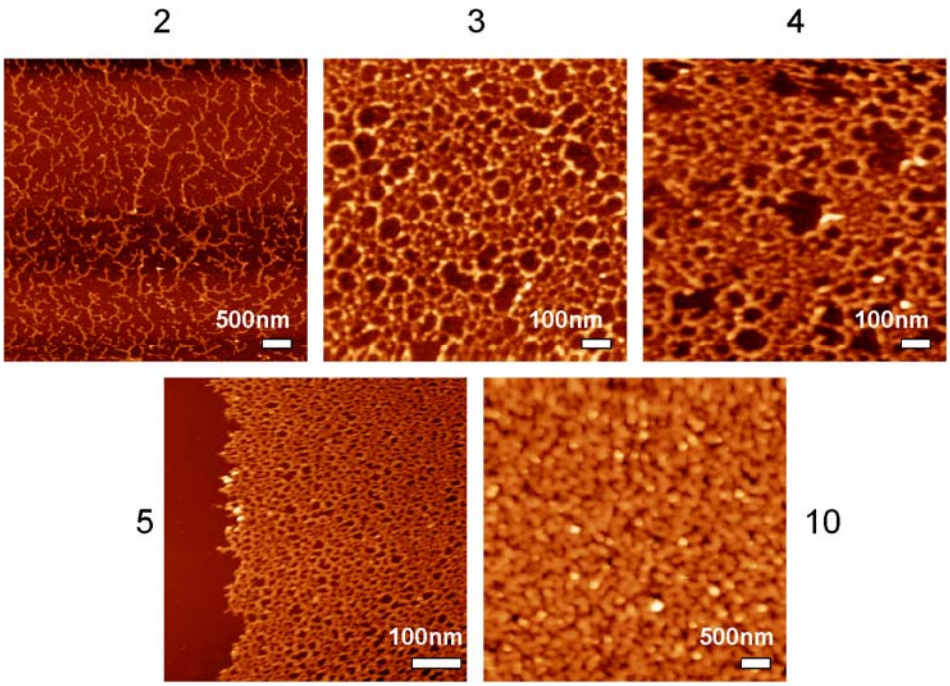


Figure 28 Fibronectin micro contact printed on silicon. The numbers in the figure indicate the protein concentration (μg/ml) in the solution used to ink the stamps.

The forces exerted by a solvent on colloidal particles during evaporation of the solvent has been employed by several groups to pattern these particles on topographically or chemically patterned surfaces[79-81]. Fan *et al*. used spontaneous dewetting of hydrophobic areas on a surface to deposit colloid suspension selectively in hydrophilic areas on the surface. Upon evaporation of the solvent, particles form patterns that are very similar to the structures observed for proteins when low particle concentrations are used. As for proteins, homogeneous particle coverage of the wetted areas is achieved when employing high particle concentrations. The drying of a protein-coated stamp varies in many ways from the described experiment. One obvious difference is that proteins adhere strongly to the stamp surface. Therefore, it requires a larger force to redistribute proteins on the hydrophobic surface than it requires to redistribute non-adherent colloids. However, we have previously established that water can certainly redistribute proteins on the hydrophobic stamp surface (see Figure 14). In addition, the surface from which evaporation occurs is hydrophobic. This will affect the contact angle of the solvent to the surface and thus the forces exerted on the proteins[81]. We therefore expected that the pattern-formation on hydrophilic surfaces will be less pronounced.

Formation of protein networks was also observed by Noemi Rozlosnik in a series of experiments carried out to study protein adsorption on hydrophobic and hydrophilic model surfaces. Silicon surfaces were coated with n-Octadecyltrichlorosilane(OTS) or N-(6-Aminohexyl) Aminopropyl-trimethoxysilane(AHS) forming surfaces with contact angles of 105° and 45° respectively. Proteins adsorbed and dried on these surfaces show clear formation

of networks on the hydrophobic OTS surfaces ensuring that networks are not caused by the micro contact printing process. As expected no network formation is seen on the hydrophilic AHS surfaces indicating that when solute contact angles are low, the capillary forces are not large enough to redistribute adhered proteins.

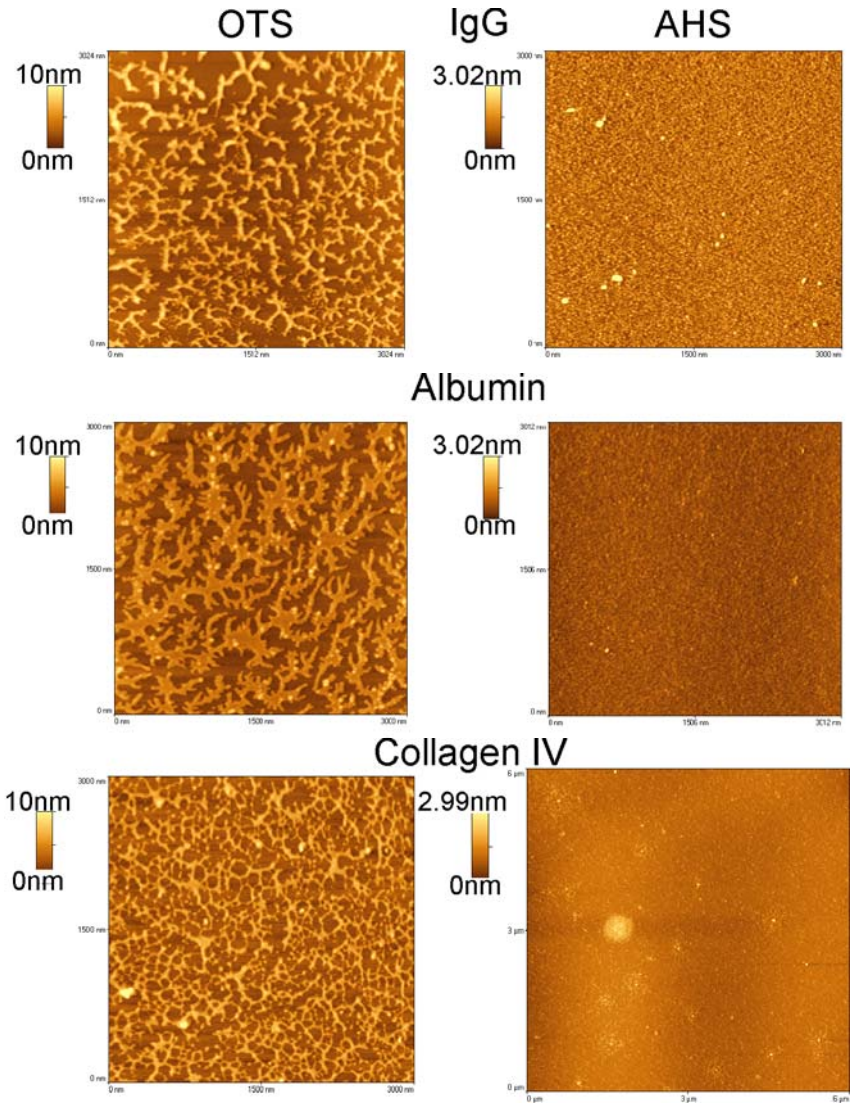


Figure 29 AFM images of model hydrophobic (left) and hydrophilic (right) surfaces coated with IgG, collagen IV and human serum albumin. All images are 3 x 3 μm^2 apart from the lower right image that is 6 x 6 μm^2. Data kindly provided by Dr. Noemi Rozlosni[82].

In conclusion, we have shown that a wide range of proteins form network-like structures when adsorbed and dried on hydrophobic surfaces. We believe this is caused by redistribution of proteins on the surface by capillary forces during drying. This may be problematic for the use of hydrophobic stamps in some of the more sensitive applications of micro contact printing, making it necessary to investigate whether the formation of these structures can be controlled.

3.11 Conclusions on micro contact printing

To our experience one of the key problems connected to utilizing micro contact printing for patterning of proteins is achieving a homogeneous coating of the stamp and thus a well-controlled protein density. It is, however, important to notice that all the presented experiments were performed using unmodified and thus hydrophobic stamps and these conclusions do therefore not encompass work where modified stamps have been employed. When structured hydrophobic stamps are utilized, it is not possible to dry stamps by spinning unless the dimension of the recessed areas is small enough to prevent the inking solution from wetting these areas. Even under these circumstances inhomogeneities in the final coating occur along the edges of stamp features. Although we tried several different approaches we did not find a method for reproducibly coating structured stamp over large areas.

However flat stamps can, reproducibly, be homogeneously coated over large areas by employing spin-drying of stamps. Thus, successful integration of micro contact printing in high throughput production should be possible by using flat stamps and adopting the strategy of subtractive inking. This method provides a large degree of freedom in terms of feature sizes and can produce both isolated and connected features.

Finally we conclude that direct micro contact printing on polymer substrates such as polystyrene and polymethylmethacrylate is not a feasible route for forming protein-patterned polymer surfaces. For reliable transfer to occur pre-treatment of substrate surfaces is required. This is important to note, as it will necessitate additional production steps, thus increasing the product end-cost. This is also the case for the subtractive inking approach, as it will be necessary to introduce extra cleaning steps in order to remove proteins from the structured subtractive inking-surface.

However, we devised a method for transferring protein from a subtractive inking-surface onto a polymeric end product without pre-treatment of the polymer surface. The remaining part of this thesis will be concerned with this method.

4 In-Mould patterning of proteins

This chapter is partially based on a manuscript submitted to Science. The chapter presents a novel method for patterning biomolecules on thermoplastic parts in the injection moulding step. These experiments were carried out in collaboration with Henrik Pranov who undertook the operation of the injection moulding machine.

4.1 Background for In-Mould patterning of proteins.

As presented in section 1.4; many methods for immobilizing patterns of biomolecules on polymeric substrates require chemical modification of the polymer surfaces. Such modification is e.g. necessary in order to achieve effective transfer of biomolecules in micro contact printing or to introduce reactive groups for Micro stamping on an Activated Polymer Surface (MAPS). It is desirable to develop a technique that avoids such modifications since this would prevent the risk of introducing reagents that are not biocompatible into the polymer matrix during the chemical modification; moreover it would eliminate expensive production steps[34].

Injection moulding is a well-established industrial process and the most commonly used method for shaping plastics[83]. The first injection moulding machines were manufactured and made available in the early 1930's. Injection moulding offers the possibility of producing parts of high geometric complexity and has a typical production cycle time of a few seconds. This, combined with the low cost of many thermoplastic polymers, is why parts with end-uses varying from train seats over children's building blocks to cell culturing dishes are produced using injection moulding.

If protein patterning could be integrated into the injection moulding step without slowing down the process considerably, this would open up a range of new opportunities since it would allow production of large numbers of parts at very low cost[16,21,84]. Apart from the appeal of low production costs, a wide range of polymeric materials can be processed in injection moulding. This makes it possible, through the choice of polymer, to optimize properties such as biodegradability and mechanical properties according to the end use of the produced parts. Furthermore, many thermoplastic materials such as e.g. polymethylmethacrylate, polystyrene and polyethylene are already widely used as biomaterials and cell culturing substrates[5,35], and products are produced by injection moulding. Therefore, if a production method utilizing these materials, injection moulding and a few additional steps to pattern proteins could be developed, this method would already largely have been examined for

introduction of cytotoxic substances. This would ease the introduction of new protein-functionalized products considerably. It would therefore be highly interesting to employ injection moulding in production of patterned biologically active surfaces.

4.1.1 Basics of injection moulding

An injection moulding machine (Figure 30 A), consists of three main parts namely the injection unit, the clamping unit and the mould (Figure 30 B). Polymer resin is fed through the hopper into the injection unit. Here the polymer resin is melted and through the nozzle it is injected into the mould that is kept at a temperature below the solidification temperature of the melt. The clamping unit holds the two halves of the mould together during injection of the melt and after solidification of the polymer the clamping unit is opened to separate the two halves of the mould and release the part.

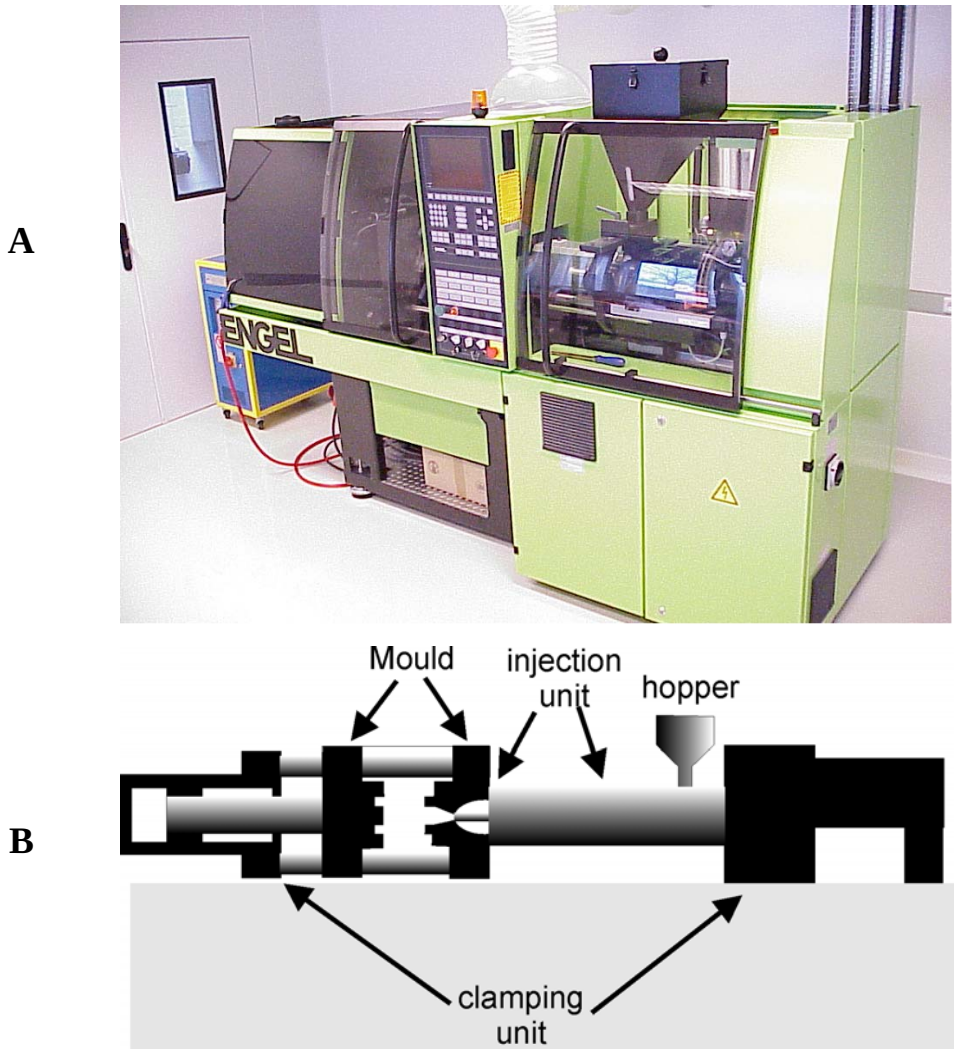


Figure 30 (A) Engel 25 tonnes injection moulding machine utilized in part of the presented experiments. (B) Schematic view of an injection moulding machine illustrating the main parts. (Adapted from[85]).

4.1.2 Transfer of colloids by micro contact printing and embossing

In 1996 Hidber *et al*. demonstrated that micron and submicron patterns of palladium colloids could be formed on silanized polymer and Si/SiO_2 surfaces by use of micro contact printing[86]. After the colloids were immobilized on the surfaces they served as catalysts in electroless deposition of copper. Electroless deposition is an autocatalytic redox reaction in which metal cations in solution are reduced and deposit on a surface as a metal layer. Generally a catalyst, such as e.g. palladium, is needed to initiate the process. Therefore no deposition will occur in surface areas where active catalyst is not present. The transferred patterns of palladium colloids thus allowed for formation of micron size metal structures. In 2002 Ng *et al.* demonstrated a one-step process that both shapes a thermoplastic part and transfers material onto the surface of the part[87,88]. They transferred patterns of palladium colloids to thermoplastic materials by use of hot embossing. A stamp consisting of silicon or stainless steel was coated with palladium colloids, heated to 150 °C and pressed against a polystyrene substrate that was heated to between 200 and 260 °C (Figure 31). Conditions for embossing were adjusted such that the cavities in the mould were only partially filled in the process. In this way, the colloids were transferred from the stamp to the polymer only in areas that were contacted by the polymer. After the colloids were transferred, nickel was deposited on the patterned surfaces by electroless deposition, which confirmed that the colloids were not buried within in the polymer but were accessible on the surface.

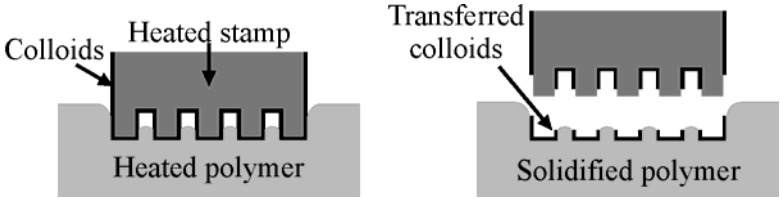


Figure 31 Schematic view of the process used by Ng et al. for transferring Pd colloids in hot embossing.

4.1.3 Transfer of colloid patterns in injection moulding

A series of experiments were carried out in order to investigate whether the approaches of Hidber *et al*. and Ng *et al*. could be combined and integrated with injection moulding. Results on the patterning of colloids will be presented, as they are used in the discussion of later results. However, only the main results will be presented since the patterning of colloids does not fall within the scope of this thesis. The experimental setup used will be described in some detail as this setup was used throughout our injection moulding experiments.

4.1.4 Mould and shims for Pd-colloids

The mould used in the injection moulding contained a replaceable shim (Figure 32). This shim was placed on a highly heat conducting backplate in which a thermistor could be mounted to monitor the temperature of the mould during injection. The mould was water-cooled and was mounted on an Engel 25 tonnes machine (Figure 30 A).

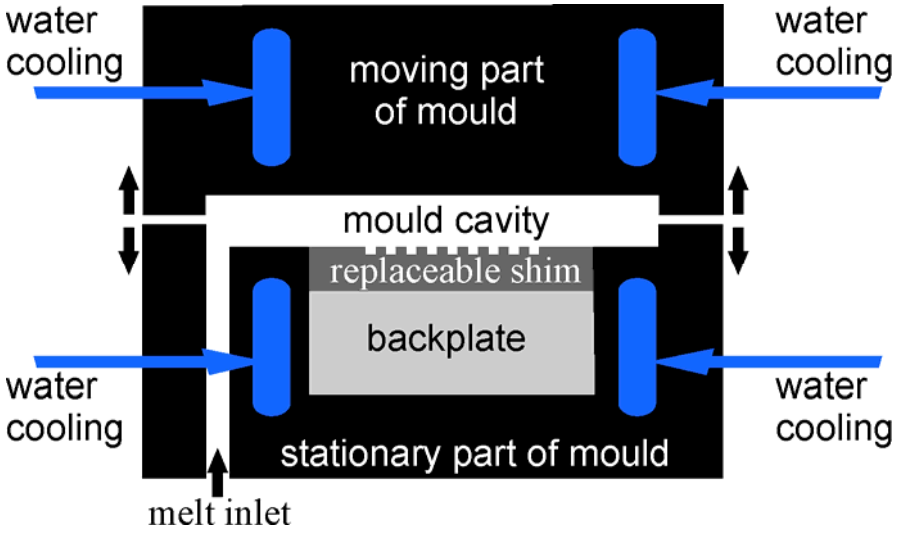


Figure 32 Schematic view of the mould used. Adapted from[89].

The shim used consisted of a 300 μm thick 39 by 44 mm^2 nickel plate with a relief pattern formed in electron beam resist. The resist layer was 400 nm thick and formed an interdigitated array consisting of 3 μm wide lines interdigitated with 16 μm wide lines. After exposure and development the used resist layer (FOx-14 from Dow Corning) formed a SiO_2 rich surface that could be used directly for injection moulding. The shim was kindly produced by Henrik Pranov.

4.1.5 In-mould patterning procedure - colloids

The in-mould patterning process is illustrated in Figure 33. The utilized Pd-colloids were kindly prepared by Dr. Mikkel Jørgensen, following the procedure described by Hidber *et al.*[86]. The colloids were suspended in heptane and sonicated one hour prior to use. A flat PDMS stamp (1 x 1 cm^2) was placed on a glass slide for support and mounted in a spin coater. Colloid solution was dripped on the stamp while it was spinning at 7000-10000 rpm. The coated stamp was carefully printed on the shim, thus transferring colloids only onto the raised FOx areas of the shim. The micro contact printing step was performed either while the shim was mounted in the mould or prior to mounting the shim in the mould. Following the printing step, injection moulding was performed using polypropylene (Inspire H715-12 Dow Plastics). In the injection moulding step the mould was clamped and polymer melt was injected into the cavity at the melt inlet. The polymer solidified in the shape of the mould cavity after which the mould was opened and the solid polymer replica was ejected from the mould,

ideally with colloids now transferred from the raised areas of the shim to the recessed areas of the replica.

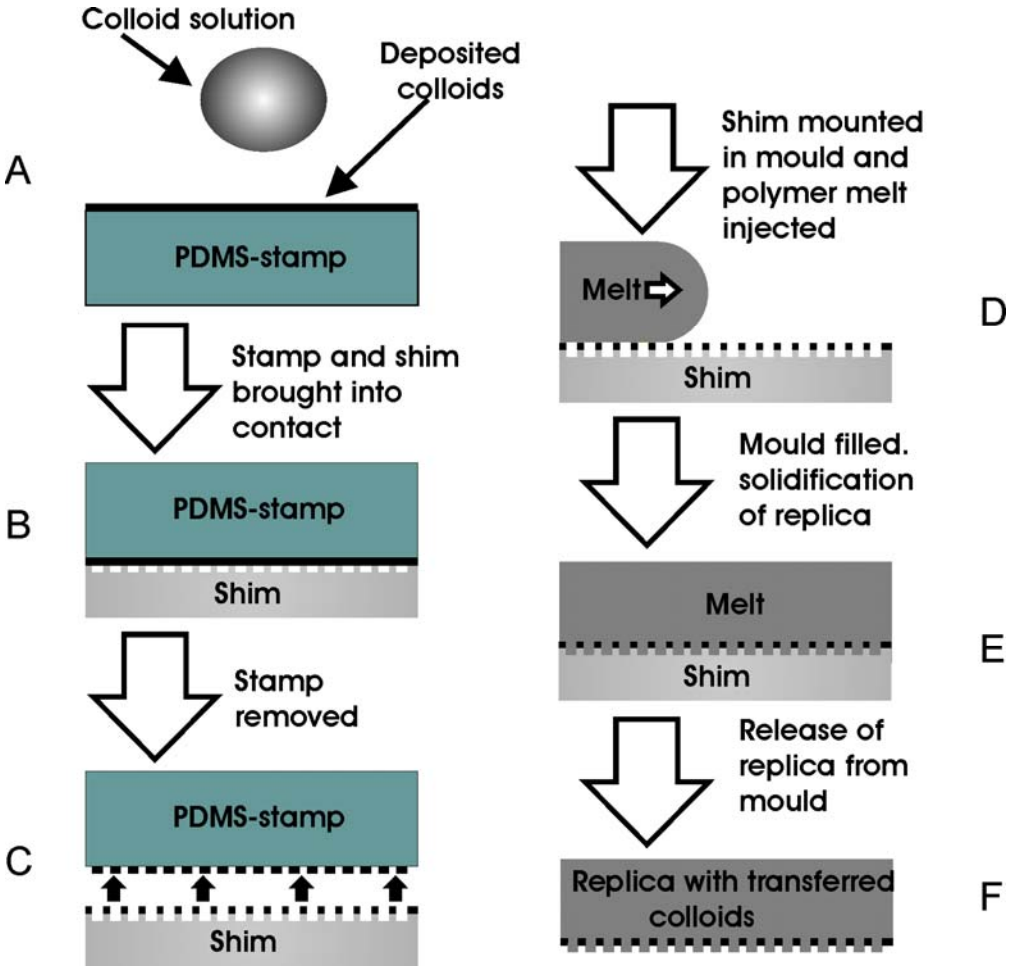


Figure 33 Schematic view of the in-mould patterning process for colloids. (A-B) A flat stamp is coated with colloids and brought into contact with the shim, (C) the stamp is removed, leaving colloids where the stamp and shim were in contact. (D) The shim is inserted into the mould and polymer melt is injected, (E) the melt solidifies, (F) the replica is released from the mould.

For electroless deposition of copper, replicas were immersed in a copper-plating bath, as described by Hidber *et al*.[86]. After a one-minute immersion, replicas were removed from the bath, rinsed in Millipore® water and dried in a flow of argon.

Bright-field microscopy images (Figure 34) of the produced replicas clearly show copper deposited in a pattern that corresponds to the resist pattern on the shim. This confirms that colloids were indeed transferred site-specifically from the shim to the replica and were not buried within the polymer.

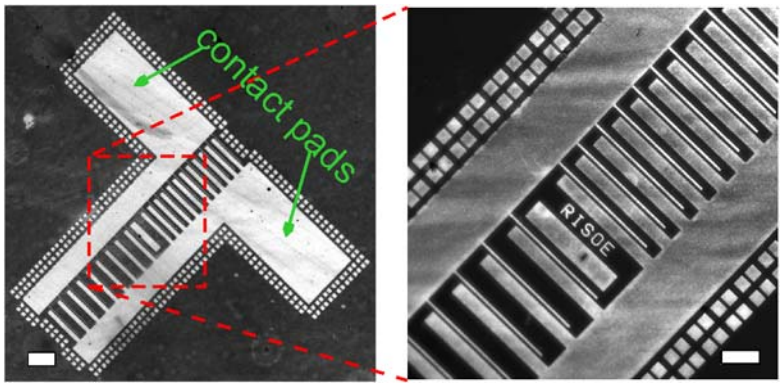


Figure 34 Bright field microscopy image of a polypropylene replica in-mould patterned with Pd colloids after electroless deposition of copper. Copper appears light while the polypropylene appears black. Scale bars are 100 and 50 μm.

Examining the replicas using AFM showed widths of the deposited copper lines of 3.1 and 16.0 μm. (Figure 35). As can be seen from the extracted height profile, copper areas are approximately 100 nm higher than the surrounding polymer. Thus the copper layer deposited is approximately 500 nm thick. (400 nm deep groove + 100 nm above). The line profile also shows a height variation of approximately 50 nm across the 16 nm wide lines. Since the deposition of copper is isotropic, and the deposited layer is approximately 500 nm, this shows that colloid were not deposited outside of the recessed areas. Transfer of colloids to other surface areas than the recessed areas would lead to areas approximately 500 nm above the polymer level. This is clearly not the case.

Conductive AFM measurements were carried out to examine whether the created arrays were conductive and isolated from each other. These measurements were performed in collaboration with Dr. Noemi Rosloznik. Electrical contact was established to the contact pad connected only to the 16 μm wide line array and scanning was performed using a CrPt coated tip (BS ElectriCont CrPt from Budget Sensors). As can be seen from both images and line profile the two interdigitated arrays were indeed electrically isolated from each other and the 16 μm wide lines were conductive.

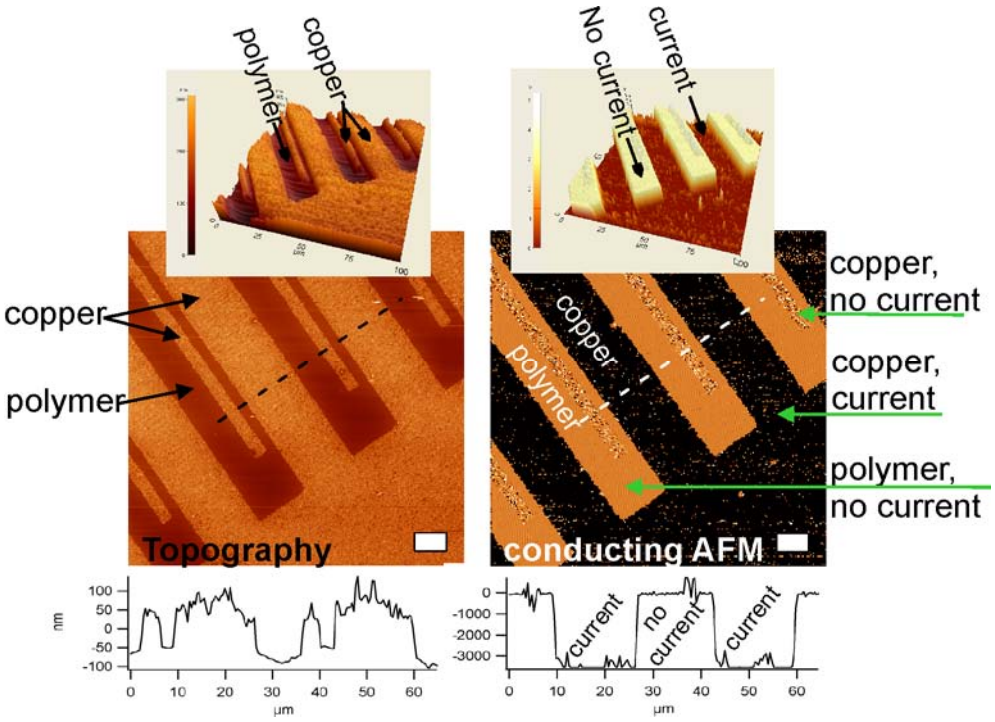


Figure 35 A polypropylene replica in-mould patterned with Pd colloids after electroless deposition of copper. Left: Topographic AFM image of the surface. Right: Conducting AFM image. Scale bars are 10 µm.

Using in-mould patterning it is therefore possible, by adjusting the bath-deposition time, to produce electrical circuits placed in recessed areas on polymer surfaces. Hidber *et al*. reported an edge resolution of about 100 nm of features formed by use of micro contact printing and electroless deposition. This resolution limit was given by the size of the copper grains. Using in-mould patterning it may be possible to form features smaller than 100 nm as the groove dimension will physically constrain the width of the structures. Furthermore, the micro contact printing step can be carried out while the shim is mounted in the mould. Therefore it should be simple to automate the entire in-mould patterning process. Injection moulding machines are routinely equipped with robotic sample handling systems, capable of performing complex procedures. Experiments performed using polycarbonate (Makrolon DP1-1265 Bayer) produced similar results indicating that it may be possible to in-mould pattern many different polymers.

The well defined transferred patterns, as evaluated by AFM, show that little or no distortion of the pattern occurs in the transfer. At first glance this may seem surprising as the melt fills the cavity with an overall flow that moves parallel to the surface of the shim (Figure 36).

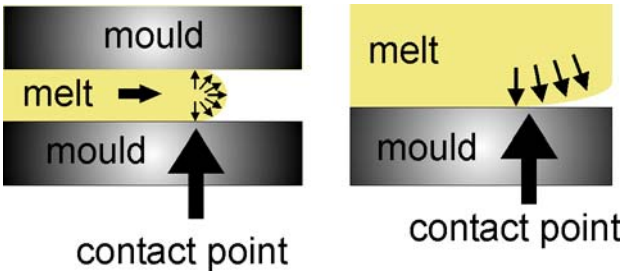


Figure 36 Schematic illustration of the flow of the melt as it distributes in the mould cavity.

However, Gramberg *et al.* have modelled the flow of an incompressible liquid between two parallel planes finding that as the liquid approaches the mould planes the axial and transverse velocity of the liquid approaches zero[90]. This implies that the melt does not slide across the surface of the shim as it fills the mould cavity and therefore no distortion of the pattern is seen.

4.1.6 Temperature development of the mould during injection moulding

Thermoplastic material is injected into the mould at temperatures well above the melting temperature of the polymer. Melting temperatures for some commonly used thermoplastics are listed in Table 2. The high temperatures of the melt indicate that if one were to attempt to transfer proteins in the manner described for colloids, these would most likely heat-denature in the process. However, modelling of the temperature development of shim and melt during injection moulding surprisingly predicts that a nanometer scale thick skin layer of melt initially at 300°C cools to a temperature of 5-10°C above the mould temperature in less than one microsecond[89,91].

Polymer	**Melting temperature °C**
Polystyrene	240
Polycarbonate	220
Polypropylene	160
Polymethylmethacrylate	140

Table 2 Examples of melting temperatures for some commonly used thermoplastics. Melting temperatures of commercial polymers vary markedly depending on chain length and additives used in the specific brand.

This suggests that if the mould temperature is maintained below 30 °C, proteins patterned onto the shim might be transferred from shim to polymeric replica while retaining a sufficient amount of their higher level structure to preserve enzymatic activity, permit immunoassays, induce cell adhesion and preserve specific binding of ligands. This possible method for patterning proteins was investigated and the results are presented in the following sections.

4.2 Experimental set-up and procedures – proteins

4.2.1 Mould and shims for proteins

Experiments on in-mould patterning of proteins were performed using the same mould that was used for in-mould patterning of colloids (Figure 32). Several different shims were used in the experiments that will be presented. All shims were 39 by 44 mm^2 with a thickness of 300 μm. Flat shims had either a pure nickel surface or were coated with FOx e-beam resist. This coating was formed by spin-coating FOx-14 on a nickel surface and hard baking for one hour at 350 °C. These coated surfaces were advantageous when performing experiments using fluorescence labelled proteins as they did not quench the fluorescence signal, as did the bare nickel shims. No difference was at any point observed between results obtained using nickel and FOx surfaces and therefore no distinction will be made between the two.

A structured nickel shim was used for all experiments requiring a structured shim. This shim contained 25 1x1 mm^2 areas with surface relief grid structures of varying width and pitch. Since not all areas were used in the experiments, width and pitch of gridlines in the utilized areas will be described in connection with the performed experiments. The grid structures were raised 200 nm relative to the base of the shim. This shim will hereafter be referred to as the structured nickel shim. The structured nickel shim was kindly produced by Dr. Nikolaj Gadegaard at Glasgow University using the same fabrication methods as described by Gadegaard *et al*.[92].

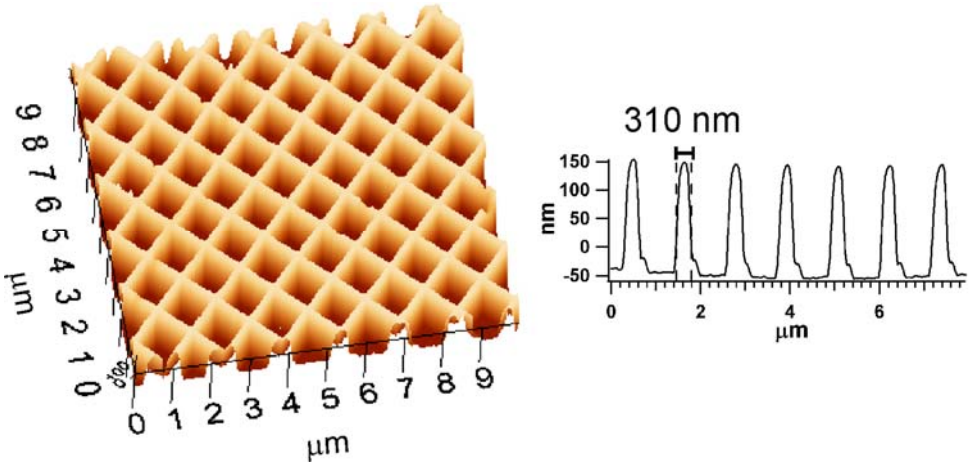


Figure 37 AFM image of an area on the patterned nickel shim containing 310 nm wide ridges separated by 1 μm.

4.2.2 In-mould patterning procedure

The in-mould patterning process is illustrated in Figure 38. A flat or structured PDMS stamp was inked with the relevant protein, carefully brought into conformal contact with the raised areas of a topographically structured or flat

shim and removed again leaving proteins where the stamp and shim were in contact (Figure 38 A-C). This was either done prior to mounting the shim in the mould or while the shim was mounted in the mould. If printed outside the mould, the shim was mounted in the mould, polymer melt was injected and after solidification the replica was released from the mould (Figure 38 D-F). All experiments were carried out using PDMS Sylgard 184 stamps for micro contact printing.

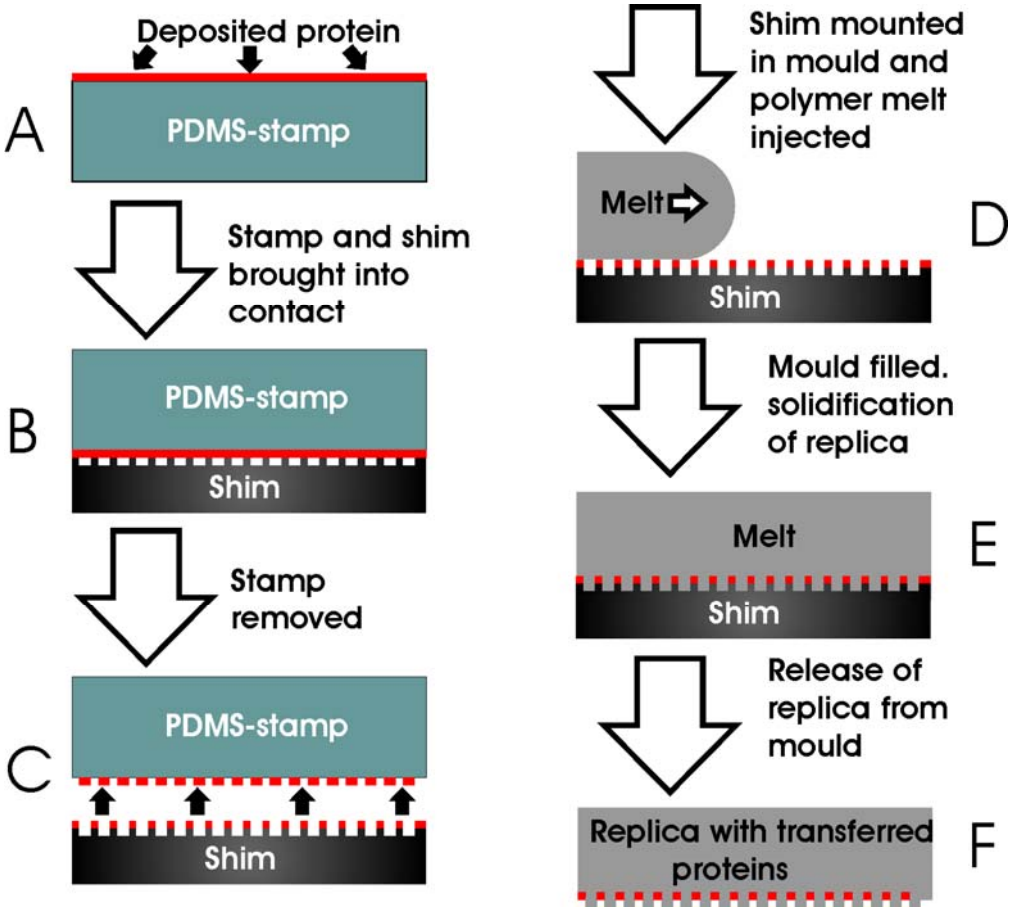


Figure 38 Schematic view of the in-mould patterning process. (A-B) A flat stamp coated with the desired protein is brought into contact with the shim, (C) the stamp is removed, leaving protein where the stamp and shim were in contact. (D) The shim is inserted into the mould and polymer melt is injected, (E) the melt solidifies, (F) the replica is released from the mould.

The mould was cooled to between 17 and 26 °C and during the injection moulding the temperature at the thermistor in the back plate increased 5-10 degrees, never exceeding 35 °C. Between each production cycle the shim was cleaned in chromesulphuric to ensure that no protein from the prior cycle remained on the shim.

Three different configurations were used when patterning proteins (Figure 39). Micro contact printing a flat shim with a patterned stamp (Figure 39 A) creates a protein pattern on the shim corresponding to the raised areas on the stamp. Injection moulding produces a polymeric replica without topographical structure

and the same protein pattern as was on the shim. Micro contact printing a structured shim with a flat stamp (Figure 39 B) results in protein coating of all raised areas on the shim. Injection moulding produces a replica with the inverted topographical structure of the shim. The transferred proteins will be located in the recessed areas on the replica. Micro contact printing a structured shim with a structured stamp (Figure 39 C) coats the raised areas of the shim within a pattern corresponding to the raised areas on the stamp. Injection moulding produces a replica with the inverted topographical structure of the shim. The protein pattern is as on the shim with all protein located in recessed areas on the replica.

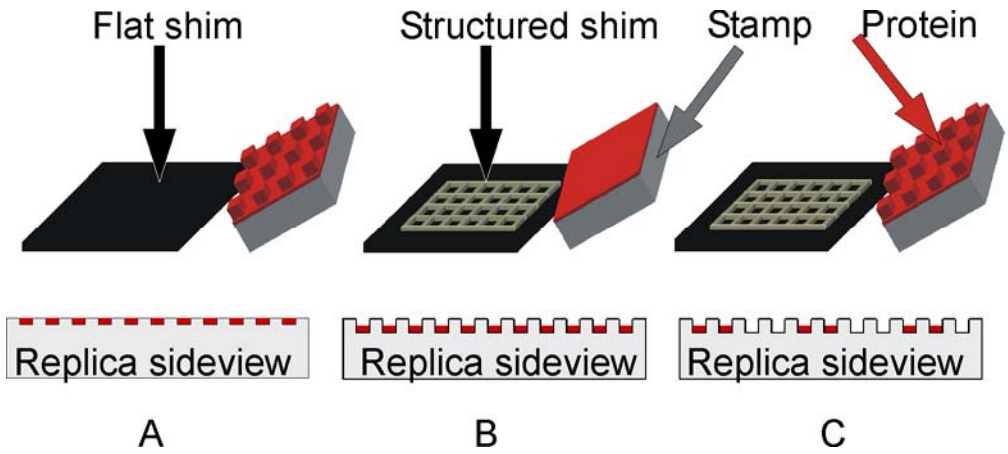


Figure 39 Schematic view of the different stamp-shim configurations used for patterning proteins on the shims. Side views of the replicas illustrate the resulting protein patterns. (A) Structured stamp on a flat shim. (B) Flat stamp on a structured shim. (C) Structured stamp on a structured shim.

Using the different combinations of structured or flat shims and stamps was necessary to, for example, allow separation of topographical and chemical signals on the replicas when this was desirable. The motivation for using each of the various configurations of patterned or flat shims and stamps will be specified in the description of the experiments where each of these have been used.

4.3 Demonstrating spatially selective transfer

4.3.1 Transfer of IgG

The feasibility of spatially selective transfer of protein from the shim to the polymer replica was demonstrated using the fluorescence labelled Alexa Fluor 546 goat anti-mouse IgG (H+L) from Molecular Probes (IgG-Alexa). Using fluorescence labelled proteins permitted direct imaging of transferred protein patterns by use of fluorescence microscopy. Prior to performing transfer experiments, a drop of the protein solution was dried out on a glass slide and heated to approximately 250 °C by placing it on a hotplate. The slide was then examined in fluorescence microscopy. We observed a clear fluorescent signal ensuring that if heating of the protein were to occur in the transfer, this would not

affect the fluorescence and therefore it would still be possible to locate transferred protein using fluorescence microscopy.

Using flat stamps, IgG-Alexa was micro contact printed onto the structured nickel shim (configuration B in Figure 39). Stamps were inked by placing a 200 µl drop of a 25 µg/ml solution of the labelled IgG-Alexa on the stamp surface. After 10 min incubation the stamp was mounted on a glass slide in a spin coater. 2 ml Millipore® water were added and the stamp was spun dry (2500 rpm, 10 s). Immediately after drying, the stamp was placed on the structured shim. IgG-Alexa was micro contact printed onto areas containing a surface relief grid structure consisting of either 3.100 µm wide lines separated by 10.000 µm or 310 nm wide lines separated by 10.000 µm. After mounting the shim in the cooled mould, injection moulding was performed using polymethylmethacrylate (Diakon TD 525 from Lucite). Fluorescence microscopy and Atomic Force Microscopy (AFM) images of the resulting replicas clearly showed that transferral of IgG-Alexa was feasible and that the grid line pattern produced by micro contact printing the raised areas of the shim was preserved in the transfer (Figure 40 A-B).

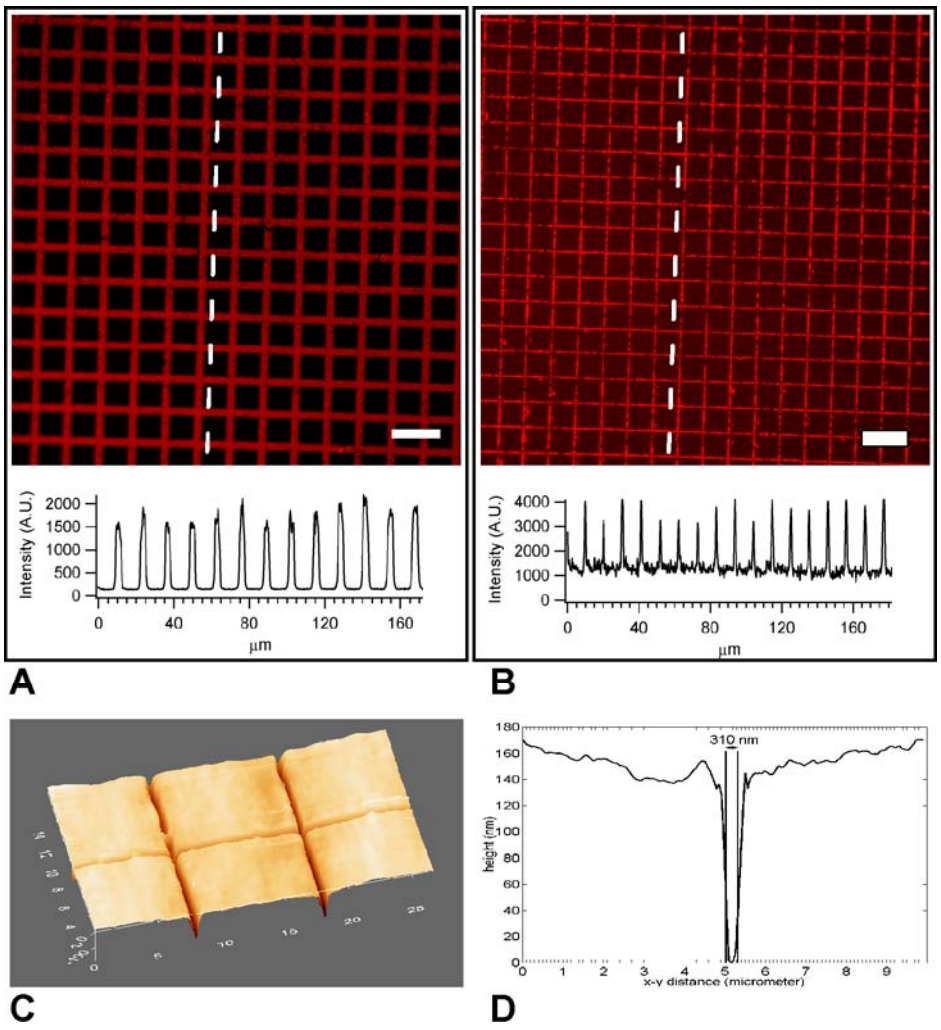


Figure 40 (A, B) Fluorescence microscopy images of IgG-Alexa in-mould patterned in respectively 3.1 µm and 310 nm wide trenches in

polymethylmethacrylat replicas. Below: line profiles showing intensities along the paths indicated by the dashed lines in the image. (C) AFM image of the replica shown in (B). (D) Height line profile showing a width of 310 nm of the trenches. Scale bars 20 μm.

Average distances between grid lines on the two replicas were determined from intensity line profiles along the dashed lines. These distances, listed in Table 3, match the nominal periods of the structures on the utilized shim very well.

Replica	Centre to centre distance on shim	Centre to centre distance on replica
A	13.100 μm	13.123 μm ± SD 0.139 μm (n=12)
B	10.310 μm	10.316 μm ± SD 0.088 μm (n=15)

Table 3 Centre to centre distances between grid lines measured on fluorescence microscopy images on replicas patterned with IgG-Alexa.

The approximately 300 nm resolution of the laser scanning microscope, when operated with fully open pinhole, prevents exact evaluation of the width of the transferred lines of protein. There are, however, several indications, mentioned below, that the line widths of the transferred protein patterns correspond to the widths of the grid lines on the shim.

The replica surface topography visualized by AFM shows a grid of trenches that is equivalent to the surface relief grid structure of the shim (Figure 40 C,D). This is consistent with the transfer of IgG-Alexa to recessed surface areas. Furthermore, results presented in section 4.1.5 show that palladium colloids micro contact printed onto raised areas of a shim are transferred only to recessed surface areas of the replica. This, along with the highly preserved regularity of the IgG-Alexa patterns strongly suggests that the IgG-Alexa has been transferred only onto the recessed surface areas of the replica and thus the widths of the transferred lines on replica B are most likely 310 nm.

IgG-Alexa was also successfully in-mould patterned onto polycarbonate (PC), polystyrene (PC) and polypropylene (PP).

Having confirmed the feasibility of transferring patterns of antibodies onto polymeric replicas by injection moulding it still remains to examine whether proteins transferred in this way can be of any use. This first of all requires that proteins retain a sufficient amount of their higher-level structure to preserve their biological functions. Furthermore, a too high degree of encapsulation of the proteins by the polymer could be another possible hindrance to the worth of the technique, since this would render active sites inaccessible to cells and ligands.

We selected three types of biological function to be probed after transfer, namely cellular recognition of an extracellular matrix protein (fibronectin), enzymatic activity (horseradish peroxidase) and lock-and-key immobilization (avidin-biotin). These data will be presented in the following three sections.

4.4 Patterning cells by in-mould patterning of cell adhesive proteins

4.4.1 Possible applications for in-mould patterning of cell adhesive proteins

If adhesion promoting biomolecules could be in-mould patterned while retaining the ability to induce cell adherence, this would provide a fast and inexpensive method for producing substrates containing defined cell adhesive regions. A wide range of possible applications can be envisaged for such a method.

One obvious application would lie in the field of high-density cellular microarrays for high-throughput drug testing[93]. New possibilities would be offered by the option of patterning proteins in recessed areas. Patterned or homogeneous coatings of adhesive proteins could be placed on the bottom of wells like e.g. standard well-plates. This would allow easier addition of substrates to be analyzed (Figure 41 A) compared to planar substrates that are patterned or homogeneously coated with cell adhesive proteins. By incorporating micro wells within larger wells, see Figure 41 B, smaller cell clusters could be analyzed. This would still preserve the possibility of adding substrates to wells using equipment standardized to operate with well plates. The ability to produce such parts would ease large scale testing of compounds on single cell or cell cluster arrays.

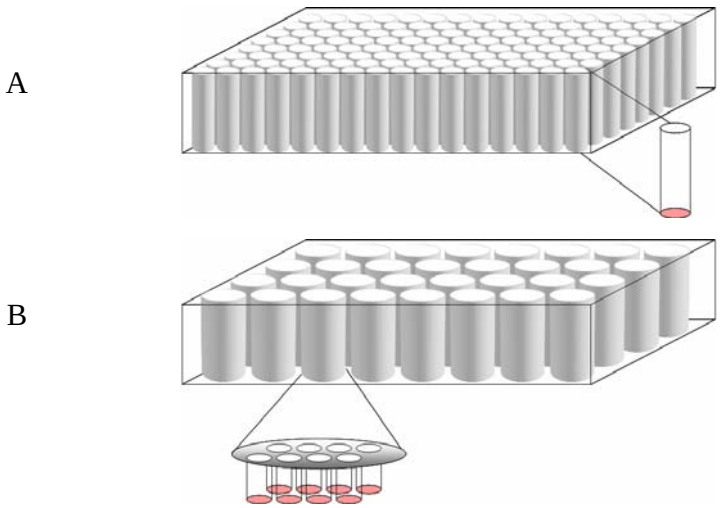


Figure 41 Schematic view of well plates in-mould patterned with protein, indicated in red, (A) either in large wells or (B) in smaller wells within larger wells, situated at the bottom.

Another possible application lies within micro-electromechanical devices utilizing microfluidic channels for in vitro analysis of cells and cell based biosensors. An important characteristic of these systems is the minimization of reagent volumes combined with high throughput and efficiency[94]. Analysis of anchorage dependent cells in such systems requires immobilization of the cells within the channels. This can be accomplished by incorporating cell adhesive areas on the surface of the microfluidic channels. Formation of cellular arrays within the microfluidic channel is then attained prior to the addition of analytes by first allowing cells to adhere and spread on the adhesive patches and then rinsing off non-adhered cells from the uncoated areas. Patterning of proteins in microfluidic channels has been done previously using e.g. laminar flow patterning[95] or soft lithography[96]. This patterning could, however, also be achieved using in-mould patterning. An added advantage of employing in-mould patterning for this application is once more the possibility of patterning recessed areas. Khademhosseini *et al.* produced microwells with a depth of approx 25 μm within microfluidic channels[97]. Despite flow of medium in the channels, cells were captured and remained within the wells. For analysis of adherent cells, wells with adhesive bottoms were produced and cells adhered and spread on these. By docking cells within wells, exposure to shear forces can be avoided during analysis in the microfluidic system. This is an advantage since shear forces have been found to modify cell behaviour[98]. The process used by Khademhosseini *et al.* consists of several production steps. Microwells are moulded on top of glass substrates and wells with adhesive bottoms are created by forming wells that expose the underlying glass substrate. Using in-mould patterning such surfaces could be produced entirely in plastics using a simple, inexpensive and rapid manufacturing process[21]. Furthermore, various adhesive molecules could be immobilised on the bottom of wells optimizing the surface for the specific cell type to be examined[84]. Producing microfluidic systems by injection moulding is desirable because production is simple and inexpensive when compared to e.g. glass or silicon based systems. Furthermore, there is a wide range of plastic materials available, which makes it possible to optimize material properties to the end use of the system[21].

Fibronectin is a large extracellular matrix protein that promotes the adhesion of many mammalian cell types and therefore it was selected to illustrate the methodology's viability.

4.4.2 Verification of transfer by Immunolabelling

Fibronectin is three times larger than the antibodies transferred in the experiments presented in the previous section and is well known to adhere well to both hydrophilic and hydrophobic surfaces[99]. Therefore it cannot be assumed that transfer from shim to replica will occur also for fibronectin. Experiments in this section were carried out to ensure that transfer of fibronectin occurred and that production of patterns with feature sizes in the nanometer range was feasible.

Using flat stamps, fibronectin from human plasma (Sigma-Aldrich) was micro contact printed onto the structured nickel shim (configuration B in Figure 39). Stamps with a size of app. 1 x 1 cm^2 were inked by placing a 200 µl drop of a 100 µg/ml solution of fibronectin on the stamp surface. Stamps were spun dry with initial addition of water as described for IgG-Alexa in section 4.3.1. Immediately after drying, the proteins were micro contact printed onto the structured shim. Fibronectin was printed on an area containing 100 nm wide gridlines separated by 10.000 µm and on an area containing 100 nm wide gridlines separated by 3.100 µm. After mounting the shim in the cooled mould, injection moulding was performed using polystyrene. Replicas were blocked with a 1 mg/ml solution of Human Serum Albumin (HSA) to lower unspecific binding of antibodies to the polymer surface in the immunolabelling steps. Replicas were immunolabelled by incubating first with primary antibody: monoclonal anti-fibronectin antibody produced in mouse (Sigma-Aldrich); and incubating second with secondary antibody: Alexa Fluor 546 goat anti-mouse IgG (H+L) (IgG-Alexa). Between each incubation step, replicas were rinsed in copious amounts of PBS and, after the last incubation replicas were washed in Millipore® water and dried in a stream of argon.

Fluorescence microscopy images of such replicas show a clear grid line patterns (Figure 42 A and B).

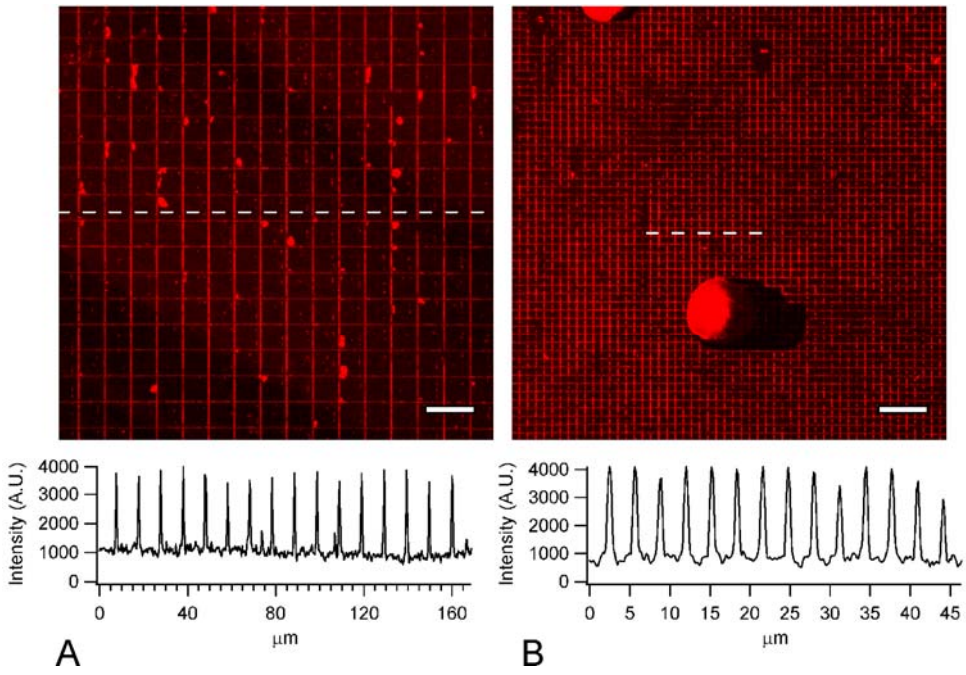


Figure 42 Fluorescence microscopy images of fibronectin transferred to 100 nm wide trenches in polystyrene by in-mould patterning. (A) 100 nm trenches 10.000 µm apart. (B) 100 nm trenches 3.1 µm apart. Line profiles show intensities along the paths indicated by dashed lines. Scale bars are 20µm

Average distances between grid lines on the replicas were determined from intensity line profiles (see Table 4). The average distances found closely matched the nominal periods of the structures on the utilized shim.

Replica	Centre to centre distance of gridlines on shim	Centre to centre distance of gridlines on replica
A	10.100μm	10.153 μm ± SD 0.151 μm (n=15)
B	3.200 μm	3.207 μm ± SD 0.082 μm (n=41)

Table 4 Centre to centre distances between grid lines measured on fluorescence microscopy images on replicas patterned with fibronectin.

The rounded bright area seen in Figure 42 B was most likely caused by water drops drying out on the surface of the stamp (see section 3.2). This could create drop shaped areas of accumulated protein with neighbouring areas on the stamp deprived of protein. When micro contact printed onto the shim, this would cause coating of both raised and recessed areas where the drop had evaporated and little or no protein transfer in the surrounding areas as observed.

We further performed a series of experiments employing structured stamps to micro contact print the structured nickel shim (configuration C in Figure 39). These experiments were carried out in order to ensure that the resulting pattern of primary and secondary antibodies was not an artefact of the nanoscale surface topography of the polymeric replica, such as e.g. preferred adhesion of antibodies in or along the edges of the trenches in the replica. By using a stamp that possessed a surface relief structure, contact and thus protein transfer should be limited to spatially separated regions where both stamp and shim exhibited elevated relief motifs. However, the nanoscale groove structure should be present on the entire surface of the replica.

The stamps used contained 40 x 40 μm^2 square raised areas separated by 40 μm in both lateral directions. Fibronectin was micro contact printed onto an area of the structured shim containing a line grid structure consisting of 310 nm wide lines separated by 3.1 μm. Stamps (app. 1 x 1 cm^2) were coated by applying a 200 μl drop of a 100 μg/ml solution of fibronectin on the stamp surface. After a 10-30 min incubation, the stamp was washed in Millipore® water, dried in a stream of air and placed on the shim immediately after drying. The shim was mounted in the cooled mould and injection moulding was performed using polystyrene. The resulting replicas were blocked with HSA and immunolabelled using primary and fluorescence labelled secondary antibodies as described for the previous experiments employing fibronectin.

Fluorescence microscopy images of the immunolabelled replicas show that binding of primary and secondary antibodies occurred exclusively in the trenches that were expected to be coated with fibronectin (see Figure 43).

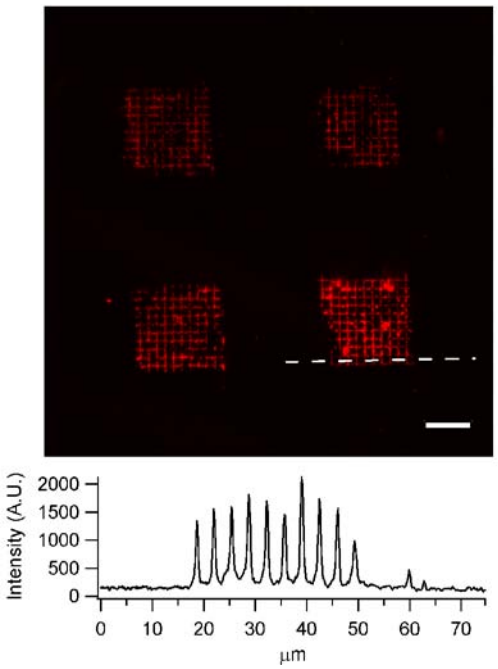


Figure 43 Fluorescence microscopy image and corresponding line intensity profile of polystyrene replica in-mould patterned with fibronectin and immunolabelled. Scale bar 20 µm.

Cell adhesion is believed to be related to binding sites placed on the 10th, 9th and 8th type-three repeats on human plasma fibronectin[100]. The primary antibody used binds to an epitope located within the 4th type-three repeat and therefore the presented results do not ensure that the adhesion related binding sites are accessible. The primary antibody used binds to both the native and the denatured-reduced forms of fibronectin. Thus, while the results confirm the presence of fibronectin on the surface of the replica they do not ensure that the transferred protein is in its native state. However, for the suggested applications, it is not critical that the protein is in its native configuration since cells both adhere, spread and grow on various denatured extracellular matrix proteins[6].

The presented experiments show that fibronectin can be transferred to the surface of a polymeric replica by in-mould patterning. Specific binding of the primary antibody shows that a sufficient amount of the transferred fibronectin is accessible to allow recognition of this specific epitope. Finally, patterns with apparent feature sizes of 100 nm or below can be created.

4.4.3 Cellular response to in-mould patterned fibronectin

This section presents experiments performed to assess the ability of in-mould patterned fibronectin to promote cell adhesion.

It is well known that surface topography can influence a wide range of cell types including nerve cells, epithelial cells and endothelial cells. Cell responses to surfaces, that present e.g. groove structures, include changes in cytoskeletal orientation, cell activation, cell orientation and increased adhesion[101]. We wished

to probe the adhesion promoting properties of in-mould patterned fibronectin. The use of structured shims in such experiments would result in replicas that present adhesion-cues originating from both surface topography and in-mould patterned fibronectin. This would complicate the interpretation of the adhesion assays unnecessarily and therefore all replicas for cell adhesion experiments were produced by injection moulding against planar shims. These were patterned with proteins by using structured stamps for micro contact printing (configuration A in Figure 39).

All adhesion assays were carried out using the HaCat keratinocyte cell line. This cell line was chosen partly because its availability. More importantly though, adhesion properties and spreading times of this cell line on traditional fibronectin-coated polymer surfaces have been studied previously in our lab using the same conditions as used when examining in-mould patterned samples[102].

4.4.4 Adhesion specificity

Cultured on e.g. polystyrene surfaces coated with fibronectin by adsorption from solution, single HaCat keratinocytes typically had a diameter of 30 μm when they were adhered and spread on the surface (Figure 44). We chose to use stamps that had larger gaps between protruding structures than the size of the single cells. This was done to reduce the risk of cells bridging the gap between adhesive areas, which would complicate the interpretation of the adhesion assays.

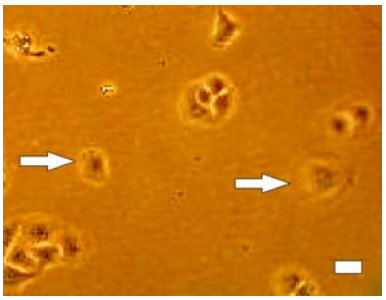


Figure 44 Phase contrast microscopy image of HaCat cells adhered on a polystyrene surface coated with fibronectin (by adsorption from solution). Single cells are indicated by white arrows. Data kindly provided by Sidse Pedersen[102]. Scale bar 20μm.

Planar shims were patterned by micro contact printing with stamps containing 100 x 100 μm^2 square raised areas separated by 100 μm in both lateral directions. Stamps were coated by placing a 200 μl drop of solution containing 45 μg/ml fibronectin and 2 μg/ml fluorescence labelled goat anti mouse IgG (IgG-Alexa) on the stamp. IgG-Alexa was added to enable direct visualization of the coated areas on the replicas. After 10 min incubation the stamp was rinsed in Millipore® water, dried in a stream of argon and carefully placed on the shim.

Cells were expected to adhere specifically via the immobilized fibronectin, however contribution to their adhesion from the co-immobilized immunoglobulin

could not be excluded. Furthermore one could argue that adhesion might still be related to the presence of topography on the replica even though planar shims were utilized. Rajnicek *et al.*[103] found that neurites exhibited contact guidance on substrates comprising groove structures with groove depths as low as 14 nm. Protein transfer is likely to result in surface areas with higher roughness and it cannot be ruled out that this may affect cell adherence.

To clarify the specificity of the adhesion we performed a parallel set of experiments in which stamps were inked using a solution containing 45 μg/ml HSA and 2 μg/ml IgG-Alexa. Incubation, washing, drying and stamping was performed as for the fibronectin-coated stamps. We did not expect immobilized HSA to induce adhesion of keratinocytes.

Shims coated with fibronectin/IgG-Alexa and HSA/IgG-Alexa were mounted in the cooled mould and injection moulding was performed using polypropylene. The resulting replicas were blocked 10 min in 5 mg/ml HSA and rinsed in Millipore® water prior to cell seeding. This ensured that observed adhesion in patterned areas would not be due to increased hydrophilicity of the replicas in these areas. Additionally, this permitted comparison of the adhesion response to HSA patterned by injection moulding and HSA adsorbed from solution. HaCaT keratinocytes were seeded at a density of app. 25,000 cells/cm^2 in DMEM supplemented with 10% foetal calf serum and 1% penicillin-streptomycin. Cells utilized were harvested from the same culture flask ensuring no difference in the condition of the cells. After one hour of incubation at 37^{o}C and 5% CO_2 atmosphere, non-adhered cells were gently removed by washing in 37^{o}C DPBS. Adhered cells were fixed using a 4% formaldehyde solution. After washing with Millipore® water replicas were dried in a stream of argon and visualized.

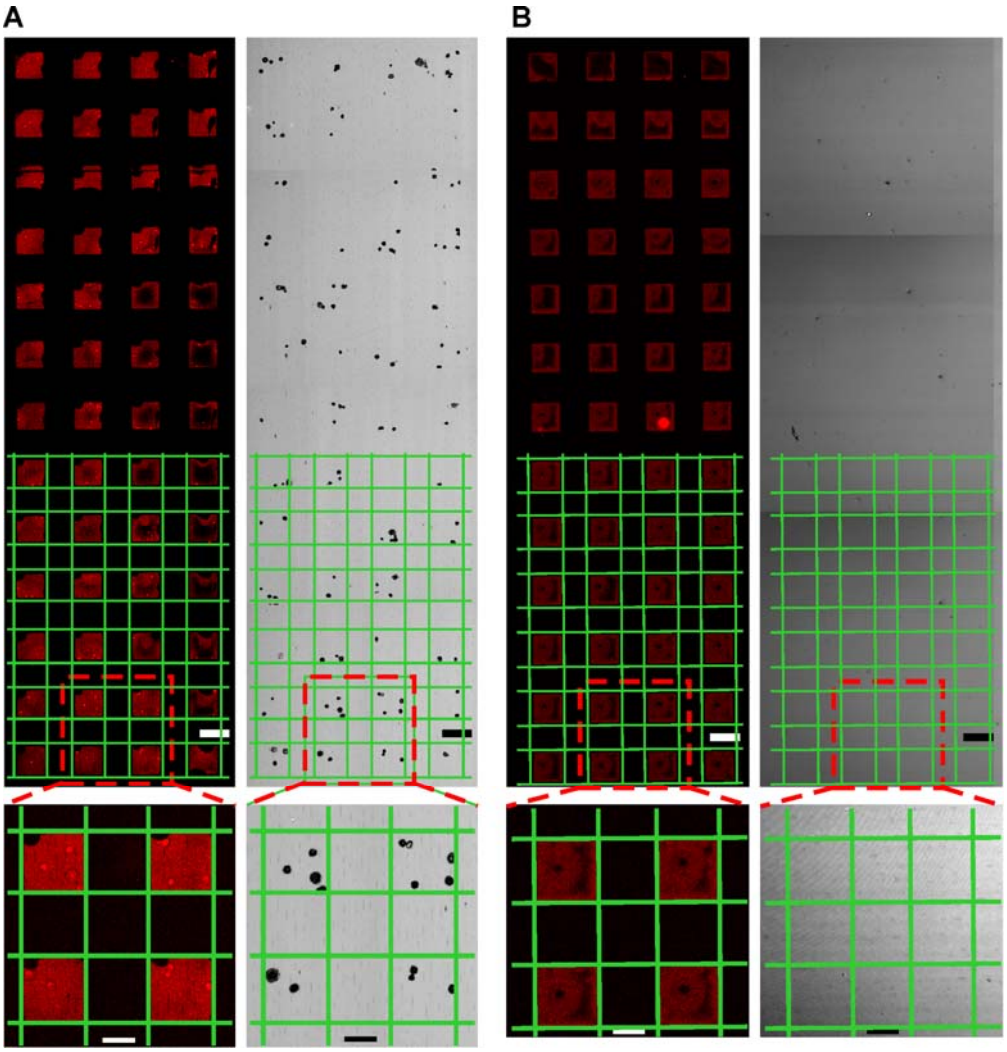


Figure 45 Fluorescence and bright-field microscopy images of protein patterned polypropylene replicas after incubation with HaCat keratinocytes. Replicas were patterned with (A) fibronectin and (B) human serum albumin. Green lines have been overlain in the lower part of the images to mark the edges of the patterned areas. Cells appear black in the bright-field images. Scale bars are 100 μm in the upper images and 50 μm in the lower images.

Fluorescence and bright-field microscopy images of the protein patterned polypropylene replicas (Figure 45) show very clear differences in cell adhesion on the two types of replicas. Analysis reveals that of the adhered cells more than 99% (N=120) are located within the fibronectin coated surface areas. Surface areas of equivalent size on the HSA patterned replicas show no adhesion of cells neither in the patterned areas containing HSA/IgG-Alexa nor in the separating regions where HSA was adsorbed after replication. The complete lack of cell adherence on this surface rules out both increased roughness of the replica in the protein patterned areas and the presence of IgG-Alexa as adhesion promoters. Adhesion on the fibronectin coated areas can therefore be attributed to specific

adhesion to fibronectin confirming that fibronectin retains its adhesion promoting abilities after in-mould transfer.

Within 3 hours of cell seeding all HaCat cells adhered on polystyrene surfaces coated with fibronectin by adsorption from solution (Figure 46). Within this period less than 10% of the cells have spread out on the surface. When examining cells that are attached on in-mould patterned fibronectin closer, it is clear that the main part of cells is already spread. This does not mean that cells spread faster on these substrates, since we most likely remove a large number of cells that are attached but not spread. However, it indicates that increasing the incubation time is likely to result in adhesion of cells in a larger percentage of the coated areas, which is desirable if these surfaces are to function as cell arrays.

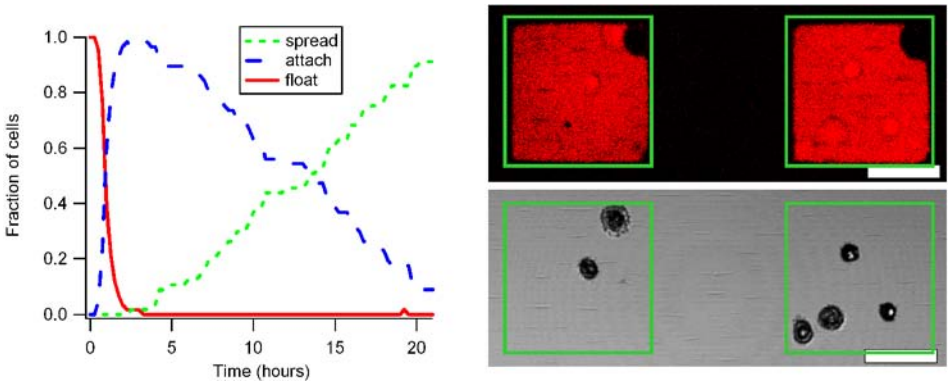
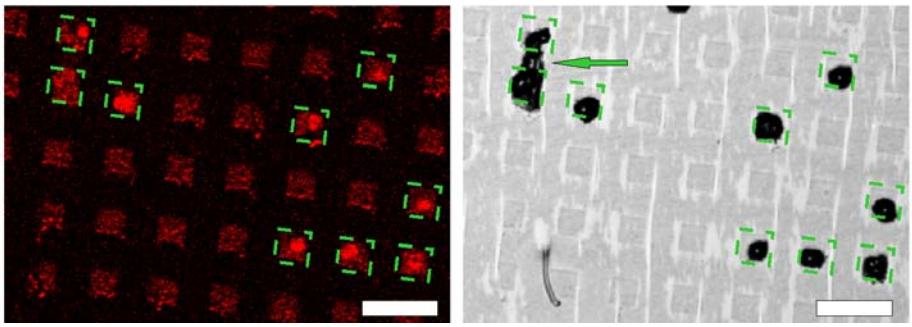


Figure 46 Left: Typical times from seeding of HaCat cells to adhesion and spreading on traditional fibronectin coated polystyrene surfaces. Data kindly provided by Sidse Pedersen[102]. Right: Fluorescence (top) and bright-field (bottom) images of cells adhered and spread on in-mould patterned fibronectin areas after one hour of incubation. Cells appear black in the bright-field image. Green squares have been overlain to mark the edges of the patterned areas. Scale bar 50 μm.

An experiment similar to the one presented above was performed using a lower fibronectin concentration, stamps with smaller feature sizes and a longer incubation time. We used the same experimental protocol as in the previous experiments. Stamps with square raised areas of 20 x 20 μm^2 separated by 20 μm were coated with a solution containing 10 μg/ml fibronectin and 2 μg/ml IgG-Alexa. Stamps with square raised areas of 40 x 40 μm^2 separated by 40 μm were coated with a solution containing 10 μg/ml HSA and 2 μg/ml IgG-Alexa. Proteins were micro contact printed onto planar shims and in-mould transferred onto polypropylene. The resulting replicas were blocked with HAS, seeded with HaCat keratinocytes and incubated for four hours after which adhered cells were fixed.

Fluorescence and bright-field microscopy images of the HSA/IgG-Alexa patterned replicas show an average of 3.8 cells/mm^2 over an area of 7.5 mm^2, whereas fibronectin/IgG-Alexa patterned replicas show an average of 104.7 cells/mm^2 over a similar area. Adhered cells are largely placed within the fibronectin coated areas but some bridging between neighbouring areas is seen

(Figure 47). As hardly any cell adhesion was seen on the HSA coated areas, bridging must be due to the lower separation between adhesive areas as compared to the 100µm structures used earlier. Comparing the number of adhered cells pr mm^2 coated area with the one hour adhesion assay shows that as expected the number of adhered cells has increased from 230 cells/mm^2 coated area to 418 cells/mm^2 coated area. Higher coverage rates could be reached by increasing the density of cells in the seeding and employing a longer adhesion time. The low background adhesion on the HSA coated areas indicates that this might be done without noticeable cell adhesion in the background.



Fluorescence Bright-field

Figure 47 Fluorescence and bright-field microscopy images of polypropylene replica patterned with fibronectin after four hours incubation with HaCaT keratinocytes. Cells appear black in the bright-field image. Cells bridging the gap between neighbouring adhesive areas are indicated with a green arrow. Green squares have been overlain to mark the edges of some of the patterned areas. Scale bars are 50 µm.

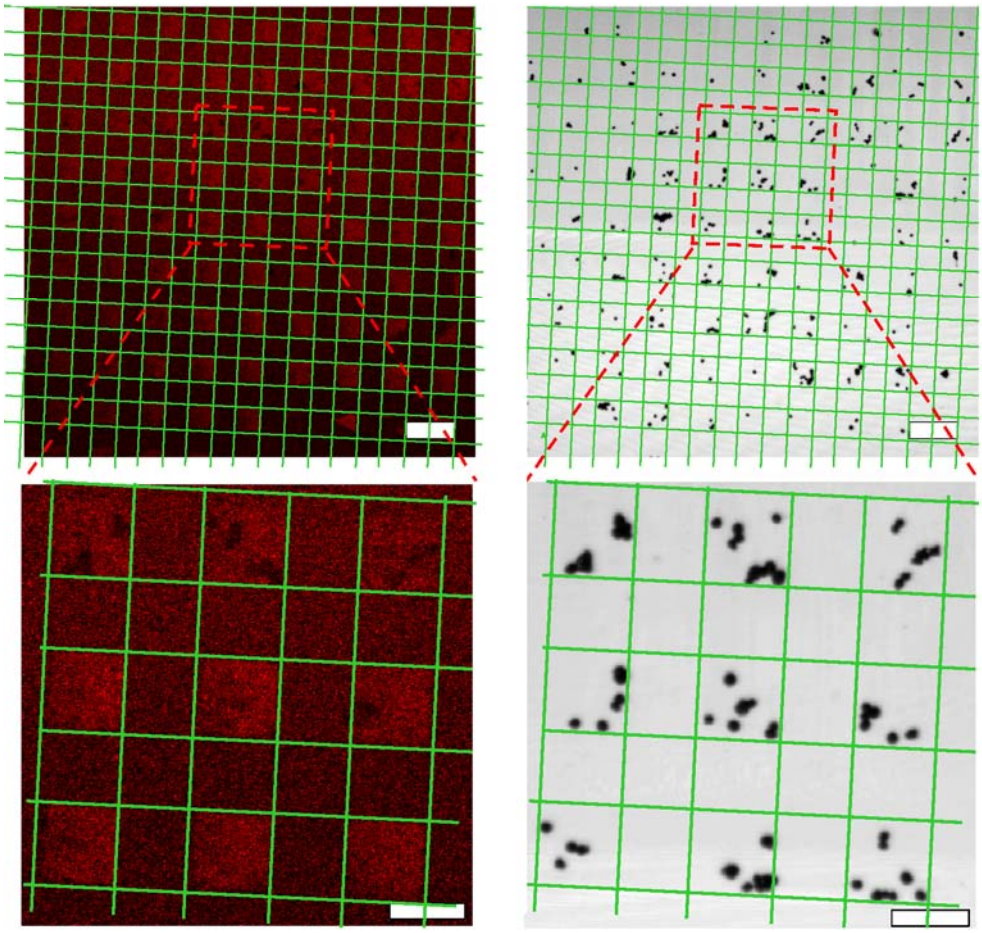
4.4.5 Adhesion and spreading without HSA blocking

The ability of cells to adhere to a given surface differs markedly between different cell types. This can be utilized for creating patterned co-cultures of different cell types. Folch *et al.*[104] used this approach to produce co-cultures of hepatocytes and fibroblasts on PS, PMMA and PC. Substrates were patterned with fibronectin or collagen I by use of PDMS microfluidic networks. After blocking with HSA, hepatocytes were seeded in serum free medium to avoid protein adsorption that could cause hepatocytes to adhere firmly outside the defined adhesive pattern. This allowed removal of loosely attached cells on the HSA coated areas. Finally fibroblasts were seeded. These cells are much more adhesive and thus they attached in the cell free HSA coated areas resulting in spatially controlled populations. Patterned co-culturing makes it possible to perform controlled studies of cell-cell heterotypic interactions that cannot be assessed through random co-cultures.

For this kind of applications it is crucial that areas that are non-adhesive to cell type number one remain so during the entire patterning procedure. However these areas must, at the same time, remain adhesive to cell type number two. If cell type number one could be patterned on replica surfaces without prior HSA

blocking of the background, this would allow coating of the remaining cell and protein free areas with a second adhesive molecule prior to seeding of cell type number two. This is of interest if one wishes to pattern a less strongly adherent cell type that requires specific adhesion cues.

We wished to examine whether HaCat cells could be patterned on surfaces, even when the HSA blocking step was excluded and cells were seeded in serum containing media. Adsorption of proteins from the media on unblocked surface areas was expected to reduce pattern fidelity by speeding up the process of cells adhering outside the defined cell adhesive patterns.



Fluorescence Bright-field

Figure 48 Fluorescence (left) and bright-field (right) microscopy images of polystyrene replicas patterned with fibronectin after incubation with HaCaT keratinocytes. Cells appear black in the bright-field images. Green lines have been overlain to mark the edges of the patterned areas. Scale bars are 200 μm in the upper images and 100 μm in the lower images.

Fibronectin/IgG-Alexa was patterned onto planar shims using the same procedure as described above. Shims were mounted in the cooled mould and injection moulding performed using polystyrene. Without prior blocking with HSA, HaCat keratinocytes were seeded on the surfaces at the same density as in the previous experiments. Conditions for culturing were the same as described

previously. After one hour of incubation, non-adhered cells were gently removed and adhered cells were fixed.

Cell adhesion analysis on fluorescence and bright-field microscopy images of the fibronectin patterned polystyrene replicas show that 96% of the adhered cells (N=230) are located within the fibronectin coated areas on the polystyrene replica. The higher number of cells, when compared to HSA-blocked replicas, is not due to increased adhesiveness of the coated areas (average number of cells per adhesive square was 2.3 and 2.8 for blocked and unblocked surfaces respectively). The higher cell-number is due to a larger analysed area.

The selective adhesion of cells to the patterned areas, even when omitting the HSA blocking step and culturing cells in serum containing media, suggests that these surfaces may also be apt for co-culturing cell types of which none are strongly adhesive. Compared to e.g. fibroblasts HaCat keratinocytes adhere and spread very quickly on fibronectin coated surfaces[102]. These adhesion experiments were carried out allowing only one hour of adhesion time. One could therefore argue that the results are not representative of the ability of these surfaces to induce patterning of other slower-adhering cell types. Pattern fidelity could be hampered by the need for increased adhesion time of cell type one. However, the presented experiments were carried out using serum containing media which should considerably speed up the process of cells adhering to the background. Despite this, excellent patterning of cells was achieved. We therefore believe that, if performed in serum free media, patterning of both slower adhering and less strongly adhering cell types is possible on this type of substrates.

The ability of in-mould patterned fibronectin to restrict cells to the protein coated areas over longer times has not yet been tested. Pattern fidelity over long times is important for some practical applications of in-mould patterning, such as e.g. cell-based drug discovery, since culture periods from 1 day up to several weeks may be required. For such applications further processing steps of in-mould patterned surfaces may be needed to prevent cells from expanding beyond the patterned regions. A well established method for preventing protein and cell adhesion on polymer surfaces utilizes PLL-g-PEG, a graft-copolymer consisting of a poly(L-lysine) backbone with multiple PEG-sidechains. At physiological pH the L-lysine backbone is positively charged and has been shown to readily adsorb on negatively charged surfaces blocking protein adsorption and cell adhesion[105,106]. PLL-g-PEG can be adsorbed on polymer surfaces after a short air plasma treatment rendering them negatively charged[107]. This technique could be applied to substrates intended for cell cluster growth as the ones indicated in Figure 41 A. Applying a slightly conical shape to the single wells would facilitate insertion of pins, physically blocking the lower part of the wells during plasma treatment, which could prevent breakdown of in-mould patterned molecules. Subsequent immersion in PLL-g-PEG would block exposed areas preventing cells from expanding into these areas.

4.5 In-mould patterning of catalytically active enzyme

4.5.1 Possible applications for in-mould patterning of bioactive molecules.

As noted, fibronectin and other extracellular matrix proteins continue to promote cell adhesion even if they are denatured. Therefore surfaces patterned with such molecules will exhibit the desired properties regardless of whether in-mould patterning causes proteins to denature or not. However, there are many other possible applications of in-mould patterning for which this is not the case.

The function of bioactive molecules such as antibodies, growth factors or enzymes is extremely dependent on their higher-level structure. Research on methods for immobilizing such bioactive molecules on solid surfaces while retaining their function is not a recent idea. Already in 1969 Weetall immobilized the enzyme trypsin on glass surfaces showing 50% of the trypsin to be active even after running a 270 hour assay[108]. One of the first methods for creating micropatterns of such molecules, that retained their biological activity, was presented in 1992 by Britland *et al.*[109]. Using photolithographic methods derived from the microelectronics industry they produced 1.5 μm wide stripes of the enzyme horse radish peroxide. One of the suggested uses for the presented new technique was "constructing new devices for incorporation into miniaturized biosensors"[109]. This has most certainly been done using the mentioned and other methods for the protein patterning step. There is a wide range of possible applications for patterns of bioactive molecules on both planar and structured substrates. However, only the application that lies within the production of microfluidic devices for performing immunoassays will be presented here. Miniaturized immunoassays are capable of faster, more precise and, due to the reduced amounts of analytes needed, cheaper analysis than typical bench-top immunoassays[20]. Several methods for immobilizing bioactive molecules have been developed. These include photoimmobilization[48,49], microcontact printing[26] and microfluidic networks[110]. In 2001 Bernard *et al.* presented a method for producing what they called "micromosaic immunoassays"[57]. Using a microfluidic network placed on a flat PDMS surface, several different antibodies were immobilized on the PDMS in 20 μm wide, parallel, separated lines. The microfluidic network was removed and the surface blocked to lower unspecific adsorption. Subsequently a second set of channels was placed on the surface, oriented perpendicular to the previously patterned lines such that each channel crossed all lines of antibodies on the surface. In this way a system, that allowed screening of solutions for the presence of several different antibodies at a time, was made. Visualization of the captured ligands could be achieved by use of e.g. fluorescently labelled antibodies. Micromosaic immunoassays combine the advantages of microarray technologies and microfluidic systems. However, this method is not well suited for mass production of assays.

There are many appealing advantages of producing microfluidic systems using injection moulding and if bioactive molecules could be incorporated in the microfluidic channels during the injection moulding step this would open up

opportunities for mass production of inexpensive standard systems e.g. for point-of-care diagnostics[20].

As noted earlier the catalytic function of enzymes is extremely dependent on their higher-level structure. Preservation of enzymatic activity during in-mould transfer would thus require that the process cause little or no permanent change in the folding of the protein around the active site. Investigating whether enzyme function is preserved could thus provide direct evidence of a high degree of preservation of higher-level structures during in-mould patterning. We chose to examine whether the enzyme Horse Radish Peroxidase (HRP) remained catalytically active after transfer by injection moulding. HRP is an enzyme isolated from horseradish roots and is one of the most commonly used enzymes in Enzyme Linked ImmunoSorbent Assays (ELISA). HRP catalyses the breakdown of hydrogen peroxide. This reaction can be coupled to the oxidation of a chromogenic compound resulting in a coloured end product that can be quantified in an ELISA reader. Thus by incubating patterned areas with solution containing a chromogenic compound it would be possible to unambiguously determine whether enzyme activity was preserved after the in-mould transfer.

4.5.2 Verification of transfer by immunolabelling

To facilitate verification of microscopically confined transfer we chose to use HRP conjugated to an immunoglobulin G (IgG/HRP); (Polyclonal goat anti-mouse immunoglobulin G/HRP, Dako cytomation). Combined with the use of structured stamps this allowed us to confirm transfer of the complex by immunolabelling the HRP-linked IgG using a fluorescence labelled antibody; (Polyclonal rabbit anti-goat immunoglobulin G/FITC, Dako cytomation) (IgG/FITC). The use of structured stamps provided reference areas internally in the patterned areas. Naturally, for the immunolabelling to be successful, it would be necessary that one of the epitopes on the HRP-linked IgG be preserved, such that it can be recognized by the fluorescence labelled antibody.

IgG/HRP was micro contact printed onto planar shims using stamps containing a raised grid pattern consisting of 20 μm wide lines separated by 20 μm in each lateral direction. Stamps (app. 1 cm^2) were coated by applying a 200 μl drop of a 10 μg/ml solution of IgG/HRP on the stamp surface. After a 10 min incubation 2 ml of Millipore® water were added and stamps were dried by spinning (2500 rpm 15 s). Immediately after drying, stamps were placed on the planar shims. Shims were mounted in the cooled mould and transferred to polypropylene by injection moulding. Replicas were blocked 5 min in a 1 mg/ml solution of Human Serum Albumin (HAS) then IgG/FITC was added and the replicas were incubated 90 minutes at room temperature. Replicas were then thoroughly rinsed in Millipore® water and dried in a flow of argon.

Fluorescence microscopy images of the immunolabelled replicas show a clear grid pattern corresponding to the protruding structures on the stamps (Figure 49). This confirms both transfer of the IgG/HRP complex and preservation, and

accessibility of epitopes for recognition on the IgG. Line profiles show a very low background intensity level. For image B in Figure 49 the average background intensity level is 19 A.U., which corresponds to the background intensity found for uncoated replicas when using the same microscope settings. This demonstrates that the transferred proteins were immobilized to washing, which is essential if surfaces are to be used in e.g. microfluidic systems.

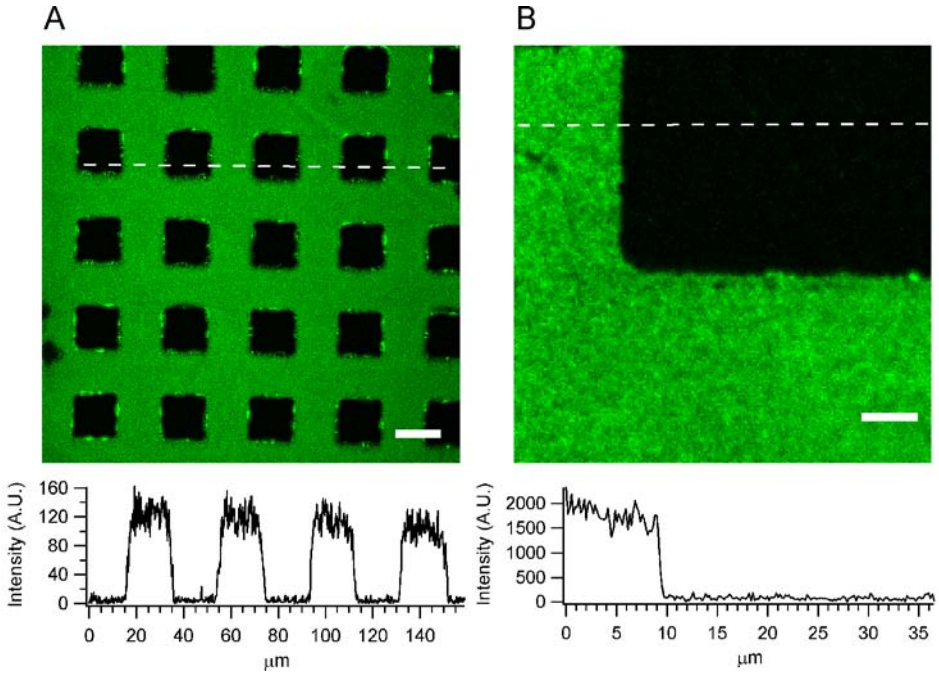


Figure 49 Fluorescence microscopy images of polypropylene replicas in-mould patterned with IgG/HRP and immunolabelled with IgG/FITC. The inserted line profiles show intensities along the paths indicated by dashed lines. Scale bars: (A) 20 µm. (B) 5 µm.

4.5.3 Test of enzymatic activity

Catalytic activity of the transferred HRP was probed on another set of replicas. In order to provide a larger coated area for analysis, flat stamps were employed to pattern the shims. Stamps (approx. 1cm^2) were protein coated using the same procedure as previously described (section 4.5.2) and were stamped on planar shims. The shims were mounted in the cooled mould and injection moulding was performed using PP. Planar shims were used in order to permit formation of reaction-wells on the replica surface for applying chromogenic substrate to the surfaces. Wells were formed, by placing PDMS masks with through holes on the patterned replicas (see Figure 50). The masks were placed such that some holes were located over IgG/HRP coated areas and others over uncoated reference areas. 100 µl of substrate was added to each well. The substrate contained 0.25 mg/ml 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Sigma-Aldrich) dissolved in the buffer solution recommended by the supplier. After a 20 min incubation the substrate from IgG/HRP coated areas and

from reference areas was transferred to a well plate and the optical density was measured in a Victor[3] (Perkin Elmer) multilabel counter.

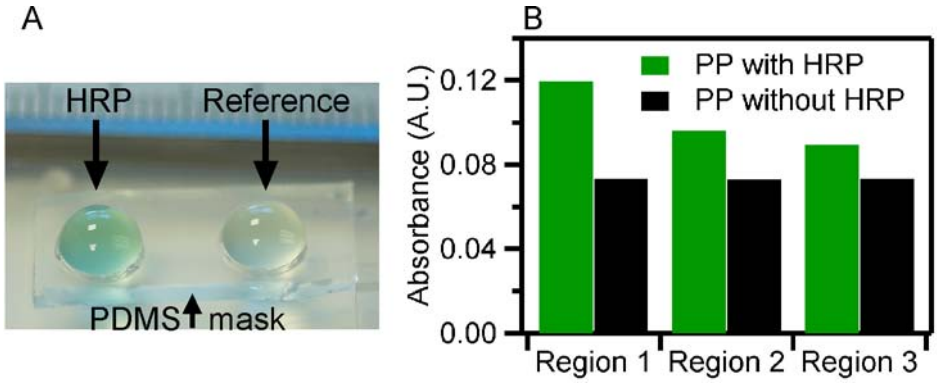


Figure 50 (A) PDMS micro wells, containing chromogenic substrate, after 20 min incubation. The wells are placed over an IgG/HRP coated area and a reference area as indicated by the black arrows. (B). The optical density of the substrate from IgG/HRP coated areas and reference areas measured after 20 min incubation.

The optical density measurements show significant enzymatic activity in all areas patterned with IgG/HRP (Figure 50). However, the activity varies markedly between the three analyzed areas, which could be due to variations in the transferred amount of IgG/HRP. To determine whether this was the case, the areas to which substrate had been added were blocked with HSA and immunolabeled with IgG/FITC using the same protocol as previously described (section 4.5.2). Subsequently all areas were imaged in fluorescence microscopy using the same microscope settings. Images show a noticeable difference in the coating density of the three areas (Figure 51). To determine whether enzyme activity scaled with the detected intensity signal we did the following. Background, stemming from well-plate and substrate-solution, was subtracted from the measured optical densities. Average fluorescence intensities in the three immunolabelled regions were determined. The background intensity, determined from the three reference areas, was subtracted. Intensities, relative to the intensity in region 1, were calculated and multiplied with the background corrected optical density measured in region 1 (Table 5).

	Relative intensities	Optical density
Region1/Region1 ∗0.046	0.046	0.046
Region2/Region1 ∗0.046	0.015	0.023
Region3/Region1 ∗0.046	0.011	0.016

Table 5 Average intensities relative to intensity in region 1, scaled to the optical density measured in region 1.

The obtained values demonstrate that the measured fluorescence signal does indeed correlate with the measured optical densities. Thus the variation in enzymatic activity between the three areas can be ascribed to variations in the amount of enzyme transferred.

The images also reveal poor and very inhomogeneous transfer on all of the three coated areas. This is most likely due to the use of a too high concentration of IgG/HRP when coating the stamps. This correlation between transfer and coating density will be discussed in chapter 5.

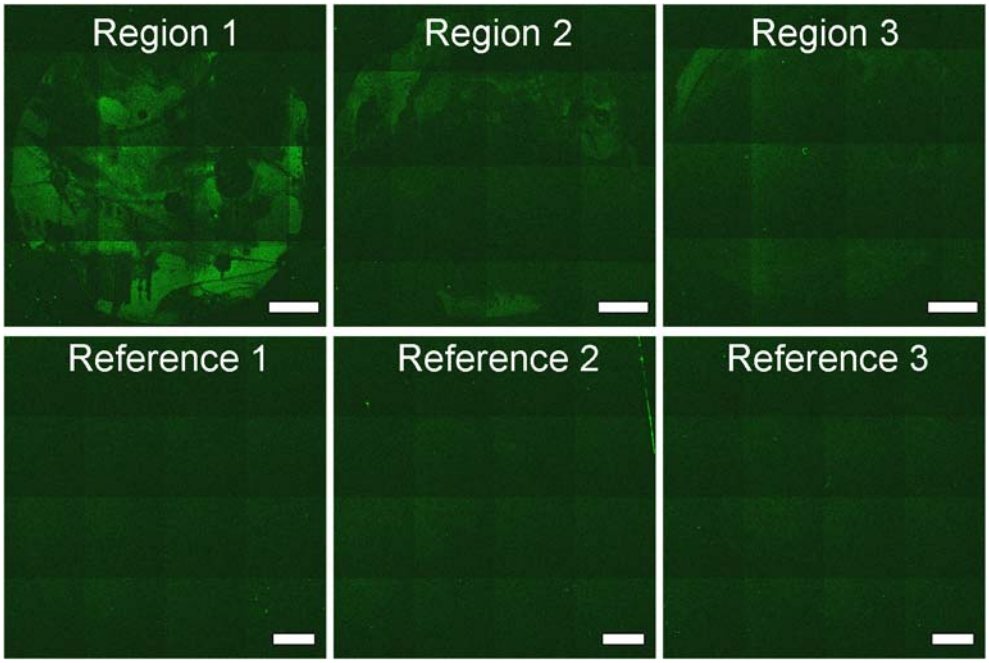


Figure 51 Fluorescence microscopy images of IgG/HRP coated areas and reference areas after addition of substrate and subsequent immunolabelling with IgG/FITC. Scale bars 1 mm.

The performed experiments do not demonstrate how large a percentage of the transferred HRP that retains its enzymatic activity or to which degree it does so. To clarify this, it is necessary to evaluate how much HRP is transferred to the surface. It is not possible to determine the amount of transferred IgG/HRP by immunolabelling with radioactively labelled antibodies, since we cannot be certain that all molecules will be detected in this way. Some molecules may be denatured “beyond recognition” or be oriented such that epitopes are not accessible to the labelled antibodies. Thus the amount of transferred IgG/HRP can only be determined by using a method that does not demand preservation of the structure of the proteins. Employing radiolabelled HRP could have been one option. In this way surfaces could be coated with equal amounts of radiolabelled HRP using in-mould patterning and adsorption from solution. This would make a direct comparison of the enzymatic activity possible. Such measurements have not yet been performed.

The preserved function of both horseradish peroxidase and fibronectin strongly suggests that secondary and tertiary structure of molecules is largely preserved in

the transfer, making in-mould patterning applicable to a wide range of thermally sensitive bioactive molecules.

4.6 In-mould patterning utilizing the avidin-biotin system

4.6.1 Indirect patterning of proteins

We believe that in-mould patterning of proteins performed in the manner described in the previous two sections may offer a pathway to producing low cost, off-the-shelf products for applications such as standardized diagnostic immunoassays and cellular arrays in various forms. However, in the field of protein microarrays, intended e.g. for use in drug discovery, direct in-mould patterning of the active protein is not likely to be applicable. In this section we will briefly go through the reasons for this and present results on an alternative strategy that may allow production of protein microarrays based on in-mould patterned plastic chips. Finally other possible strategies for producing such surfaces using in-mould patterning will be presented.

DNA/RNA microarrays are well developed and widely used for profiling gene expression and mapping mutations, thus providing information on genetic defects that may cause disease. Yet, it is the activity of proteins and metabolites that mostly affects cells and as a consequence, most drug targets are proteins[9]. Protein microarrays offer the option of screening large amounts of potential new drugs directly against their possible protein targets. Therefore they present a more direct access to develop new drugs by high-throughput screening. If effective protein arrays are to be developed, it is necessary to ensure that the biological functionality of the protein is not altered or lost in the patterning process. Nucleotides are far more stable than proteins and thus creating protein arrays is a much more challenging task. The functionality of proteins is dependent, not only on the preservation of the secondary and tertiary structure, but also on post-translational modifications, phosphorylation, and interaction with other proteins. Thus many proteins are extremely sensitive to changes in the environment, making the immobilization step crucial in the production of arrays[8,12]. In principle this is not different for the previously discussed immunoassays intended for use in diagnostics. However, in this case the patterned antibodies are immobilized with the purpose of binding a known specific ligand. It is therefore possible to ensure that the ligand in question is indeed bound by the immobilized antibodies. In contrast protein arrays are used to screen possible ligands, and therefore one does not possess knowledge of which binding sites are essential to preserve beforehand. This makes a more strict preservation of the proteins necessary.

The in-mould patterning process utilizes adsorption of proteins on the stamp surface, drying of the proteins and transfer by injection moulding. Partial denaturation is known to occur as proteins adhere to surfaces[12], and adsorption of proteins on surfaces has the inherent disadvantage that there is little or no control over the orientation of the bound molecules. This will naturally also be the case

after transfer of the proteins by micro contact printing and injection moulding. The drying step may cause further damage and finally the in-mould transfer may cause denaturation and partially embed the protein in the polymer surface thus rendering some active sites inaccessible to ligands.

It seems that the best strategy would be to pattern a coupling molecule on the surface and subsequently immobilize the active proteins on the coupling molecules via a lock and key system. The need to avoid denaturing conditions during the patterning process has led to the development of several such systems. One immobilization strategy is based on a technique developed by Wilchek and Bayer in the mid 70's[111]. It exploits the exceptionally strong binding between biotin (vitamin H) and avidin (an egg white protein). Biotin and avidin bind with an affinity constant of 1.7×10^{15} M^{-1}, which is the highest known for any protein-ligand couple found in nature. For comparison the affinity constant for antibodies and antigens lies in the range of 10^{7}-10^{11} M^{-1112}. Biotin is a 244 Da molecule and due to the relatively small size it can typically be tethered to proteins without affecting their biological function. Avidin has a molecular weight of 67 kDa and is a tetramer consisting of four identical subunits each containing a binding site for biotin. This makes it possible to employ avidin in patterning of biotinylated proteins using two different strategies. Biotin can be patterned on a substrate surface, avidin subsequently bound to the biotin and finally biotinylated proteins can be immobilized through binding to the vacant binding sites on avidin (Figure 52 A). Alternatively avidin can be patterned directly on the surface and biotinylated proteins bound to the binding sites that are not blocked by the surface (Figure 52 B). There are several commercially available products for tethering biotin to proteins by covalent attachment at specific functional groups e.g. primary amine side chains on lysine residues. Biotinylation can be done either randomly or at a specific site. The latter allows a similar orientation of all immobilized proteins, which may give higher binding activity[13].

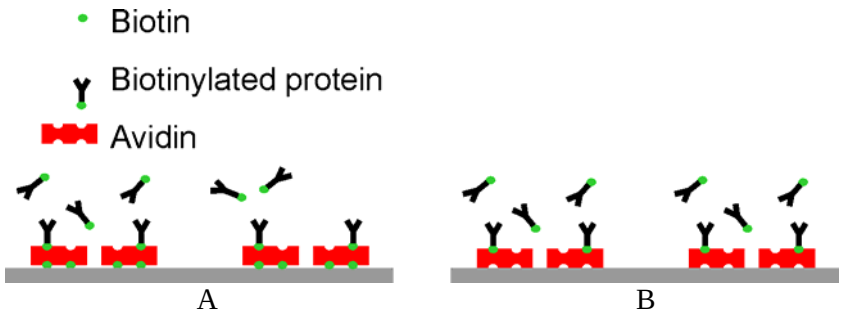


Figure 52 Cartoon illustrating immobilization strategies using (A) Immobilization of biotin followed by binding of avidin and biotinylated proteins and (B) Immobilization of avidin followed by binding of biotinylated molecules.

We chose the approach of transferring avidin directly in the in-mould patterning. On such substrates it would be possible to pattern biotinylated proteins after an

intermediate blocking step to avoid unspecific adsorption of the biotinylated proteins outside the avidin patterned areas.

4.6.2 Patterning avidin

We chose to use a fluorescence labelled avidin (avidin-TR) (Avidin Texas Red conjugate, Sigma-Aldrich) for the transfer. This facilitated verification of microscopically confined transfer.

Avidin-TR was micro contact printed onto planar shims, which provided a large coated surface area and therefore eased the evaluation of the pattern homogeneity. The utilized stamps contained a raised grid pattern consisting of 20 μm wide lines separated by 20μm in each lateral direction. The use of structured stamps provided uncoated reference areas within the patterned areas. The stamps, approx. $1cm^2$ in size, were coated by applying a 200 μl drop of a 10 μg/ml solution of avidin-TR on the stamp surface. After a 10 min incubation 2 ml of Millipore® water were added and the stamps were spun dry (2500, rpm 15 s). Immediately after drying, the stamps were placed on the planar shims. The shims were mounted in the cooled mould and transferred to polypropylene by injection moulding. Replicas were blocked 15 min in a 1 mg/ml solution of HSA, rinsed thoroughly in PBS and incubated three hours with a fluorescence labelled biotin (biotin-F) (biotin 4-fluorescein, Sigma-Aldrich). Replicas were then rinsed thoroughly in Millipore® water and dried in a stream of argon.

Fluorescence microscopy images of the replicas after incubation in biotin-F confirm that avidin-TR has been transferred to the replica and reveal co-localization of avidin-TR and biotin-F (Figure 53). This demonstrates sufficient preservation of the biotin binding site on avidin-TR to immobilize biotin-F with a high affinity.

Large area fluorescence images showed good homogeneity of the immobilized avidin and biotin over length scales of many millimetres as seen in Figure 53. The image is a tiled image recorded using the automated stage of the microscope. However, the size of the area was too large for the sample to remain within the focus plane over the entire area. This is the reason for the large intensity variation across the tiled image. An error stemming from the microscope also causes an intensity variation across each single image resulting in a higher intensity in the left side of all images. This affects the average intensities and the standard deviation calculated for the avidin-TR and the biotin-F patterns. The average intensity of the avidin pattern is 3117 A.U. with a standard deviation of only 7%. This homogeneous distribution of avidin-TR is highly promising for the possibilities of achieving well controlled densities of biotinylated molecules on in-mould patterned surfaces. The intensity of the avidin-TR signal is considerably higher than the signal stemming from biotin-F. For this reason the biotin-F pattern was imaged using the maximal detector gain of 1250 whereas the avidin-TR was imaged with a gain setting of 850. This makes a direct comparison between the standard deviations impossible as the higher gain

increases the noise in the image considerably. The average recorded intensity from the immobilized biotin-F was 1820 A.U with a standard deviation of 23 %. For comparison the average intensities of the uncoated reference areas were 207 A.U. ± SD 9% for avidin-TR and 874 A.U. ± SD 35%. for biotin-F. This suggests that the distribution of biotin-F is significantly more homogeneous than indicated by the standard deviation.

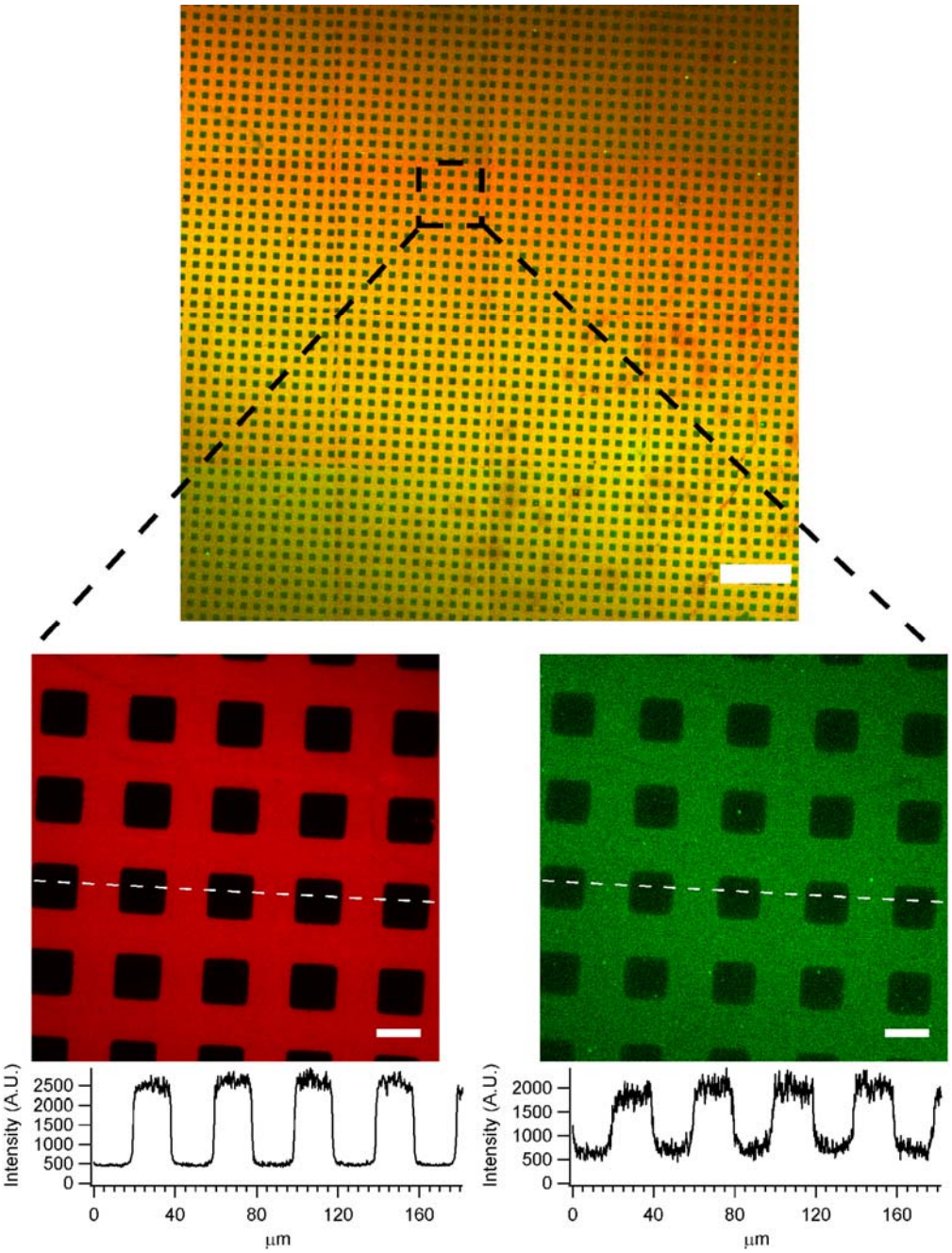


Figure 53 Fluorescence microscopy images of polypropylene replica in-mould patterned with avidin-TR and exposed to biotin-F. Line profiles show intensities along the paths indicated by the dashed white lines. Due to cross talk between the fluorophores, images were recorded using the dual track option in the software. This allows recording of the two fluorescent signals in two separate scannings. Scale bar is 200 µm in large image and 20 µm in inserts.

The replica, of which images are presented in Figure 53, was stored in air at 4°C overnight before blocking and exposure to biotin. When compared to replicas that were blocked and exposed to biotin-F immediately after in-mould patterning, no change was found in the binding of biotin to the avidin-TR. This indicates that the surfaces may remain stable for longer periods if stored in an inert atmosphere.

4.6.3 Stability of protein patterns

For in-mould patterning to be applicable in the many applications listed earlier, proteins must be immobilized on the surface. The use of a fluorescence labelled avidin allowed us to investigate the stability of the in-mould patterned avidin-TR. Replicas were produced using the same procedure as described above. However, we applied a lower concentration of avidin when inking the stamps. This was done due to the fact that we observed inhomogeneous transfer of proteins particularly when employing high protein concentrations when coating the stamps. This will be discussed in the following section.

Stamps were inked using a solution containing 1 µg/ml avidin. After transfer onto polypropylene, replicas were blocked for two hours in a solution containing 1mg/ml HSA. We chose to include this blocking step as most applications of avidin patterned surfaces are likely to incorporate a blocking step to prevent unspecific adsorption of proteins in the background. After blocking, replicas were rinsed in PBS and subsequently stored in PBS at 4°C over a four-day period. Figure 54 shows fluorescence microscopy images of a replica imaged after storage.

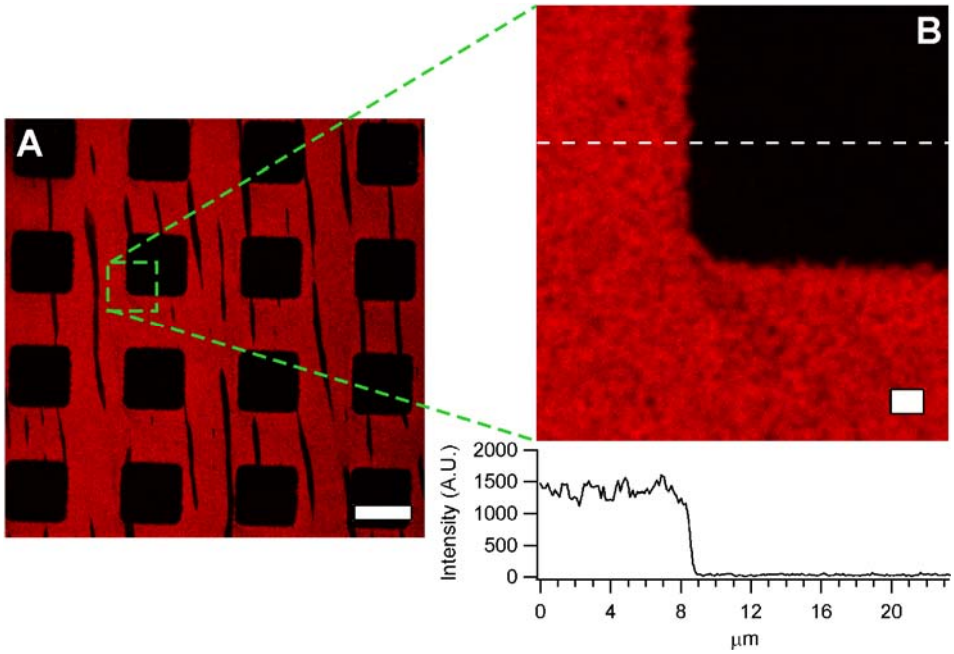


Figure 54 Fluorescence microscopy image of replica stored in aqueous media for four days. The line profile shows intensities after subtraction of background along the path indicated by the dashed white line. Scale bar (A) 20 µm (B) 2 µm.

The line profile in Figure 54 is plotted after subtraction of background intensity and reveals very low fluorescence in the uncoated areas. The background intensity was measured on an uncoated polypropylene replica using the same microscope settings as used when recording the images (Background was 399 A.U.±SD11 A.U.). Prior to background subtraction the average intensity in uncoated areas is 431 A.U.±SD12 A.U. Patterned areas show an average intensity of 1758 A.U.±102 A.U. Assuming that the coated areas contain a monolayer of avidin, which is likely to be overestimated, the intensity detected in the uncoated areas corresponds to between 0.6% and 4.4% of a monolayer. This indicates excellent stability of the transferred patterns.

The dark lines within the avidin-TR coated areas in Figure 54 are caused by flow instabilities during the injection moulding. Figure 55 shows a fluorescence microscopy image of a shim (after injection moulding) that produced a replica with structures as seen in Figure 54. From this it is clear that protein on the shim has not been smeared out, but has merely not been transferred. AFM examination of a replica with these lines of no transfer, show that the replica surface contains ripples that are between 30 and 60 nm deep. The lack of transfer is thus due to the fact that the polymer has not contacted the surface of the shim. This ripple effect has been observed at low injection speeds[89] and was ascribed to a too early solidification of the melt during the injection. In these experiments the ripples are thus likely to be due to the low mould temperature causing the melt to cool fast. When performing in-mould patterning it is therefore important to determine the minimum temperature that can be used without causing this phenomenon to occur.

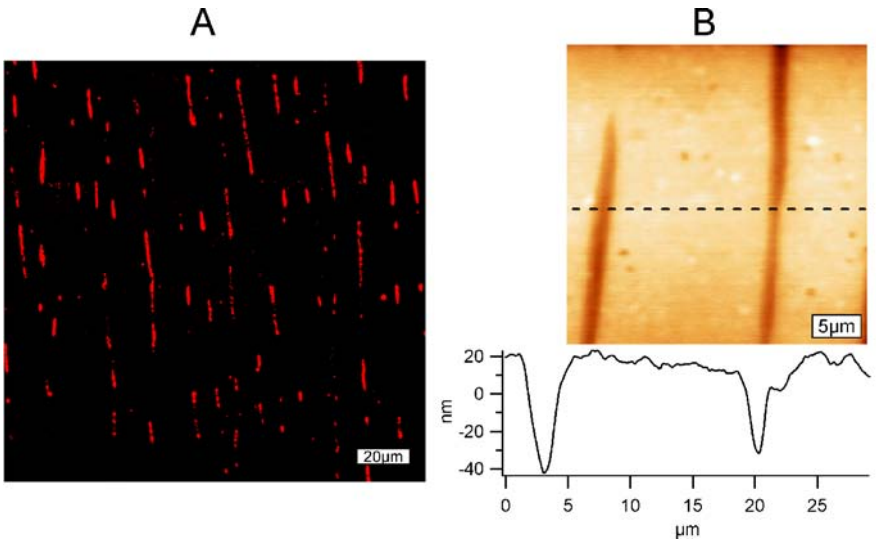


Figure 55 (A) Fluorescence microscopy image of a shim after injection moulding. (B) AFM image of replica with ripples caused by flow instabilities. Scale bars (A) 20 µm (B) 5 µm.

4.6.4 Alternative indirect patterning strategies

Another related strategy for indirect patterning of proteins could be to transfer biotinylated molecules by in-mould patterning. Recently Holden *et al.*[113]

produced patterns of biotin within assembled microfluidic channels. This was done by first adsorbing a passivating protein layer (fibrinogen) on the entire channel surface. Then channels were filled with a biotin-linked dye. After activating the dye to a highly reactive species by illumination with an Ar^+ Kr^+ laser, it bound to the passivating fibrinogen layer on the channel walls. Subsequently streptavidin-linked proteins were flowed through the channels and bound in the biotin patterned areas (see left panel Figure 56).

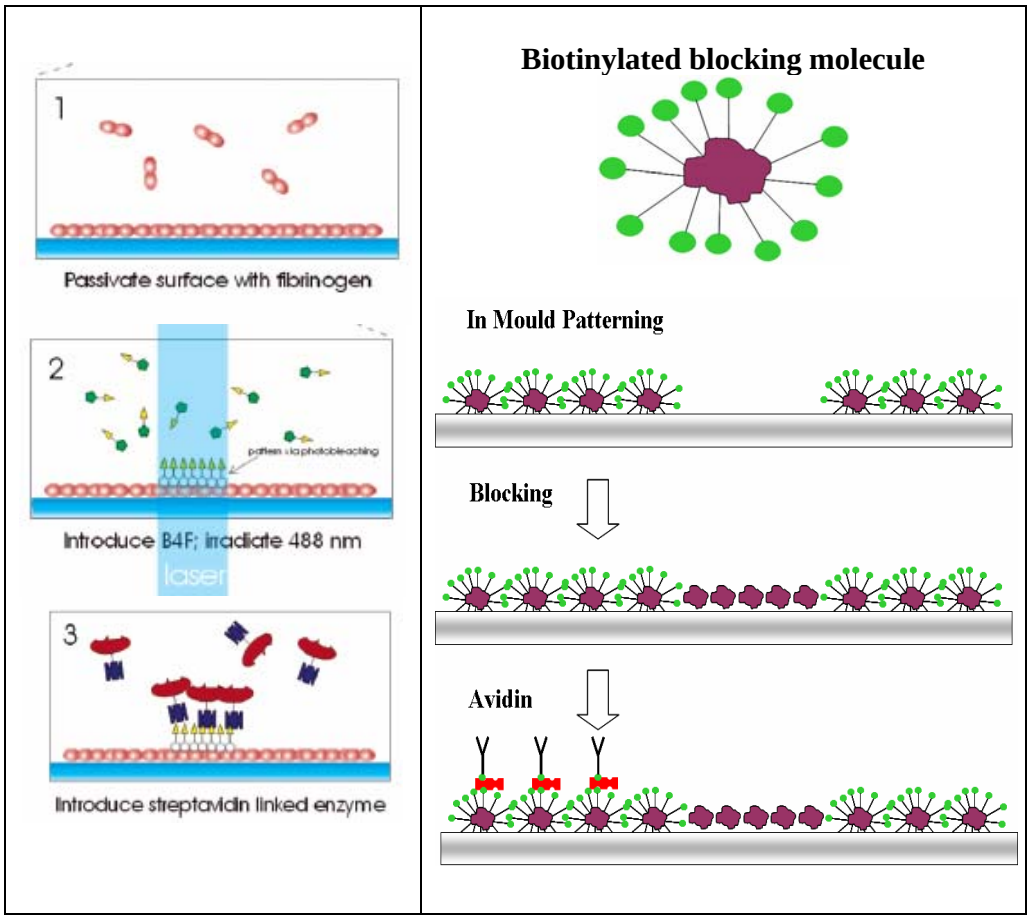


Figure 56 Left: Schematic illustration of the patterning strategy used by Holden et al. for patterning biotinylated proteins within microfluidic channels. Reprinted with permission from[113]. [Copyright (2005) American Chemical Society]. Right: Schematic illustration of the suggested method for patterning biotinylated proteins by in-mould patterning.

A similar approach could be taken with in-mould patterning, (see right panel in Figure 56). A blocking protein or another protein-repelling molecule could be biotinylized and subsequently in-mould patterned. As we have so far confirmed transfer of large proteins (fibronectin 500 kDa), smaller proteins (avidin 67 kDa) and Pd colloids it is likely that in-mould patterning can transfer other blocking molecules than just proteins. Following in-mould patterning, remaining free surface areas could be passivated by adsorbing the non-biotinylized blocking molecule from solution and finally avidin-linked proteins could be applied to bind in the biotin patterned regions.

This approach would allow preparation of surfaces for immobilization of avidin-linked proteins and, due to the high stability of biotin[114], these would be likely to have excellent storage properties. This immobilization method would furthermore move towards overcoming one of the problems pointed out by Lee and Mrksich[12]: non specific adsorption of proteins from solutions, including proteins intentionally adsorbed to block the surface, may obstruct the function of proteins immobilized on the surface due to physical blocking of the active sites. However, in the configuration presented here, proteins are located, not in plane with, but rather above the blocking proteins thus providing greater access for ligands.

5 Transfer efficiency in in-mould patterning

5.1 Protein transfer in in-mould Patterning.

For in-mould patterning to be applicable in areas such as biosensors and protein microarrays that demand a high degree of control over the density of the active molecules, it is necessary to be able to strictly control the density of protein transferred to the replica surface. As noted in the case of both IgG/HRP (section 4.5) and avidin-TR (section 4.6), large area fluorescence microscopy images of in-mould patterned replicas revealed cases of substantial variations in the intensity distribution across the transferred areas. The patterned areas were typically 1 cm^2. In all the presented experiments we coated stamps by placing a drop of protein solution on the stamp surface and spreading it with the pipette tip to cover the entire surface. Immediately after the drop was spread it typically receded from the stamp edges leaving these uncovered for the remaining time of the incubation. This suggested that, the protein density, and thus the fluorescence intensity, would be lower along the edges of the stamp than in the central area of the stamp. Fluorescence microscopy images of stamps coated with avidin-TR clearly demonstrated that this was the case (see Figure 57).

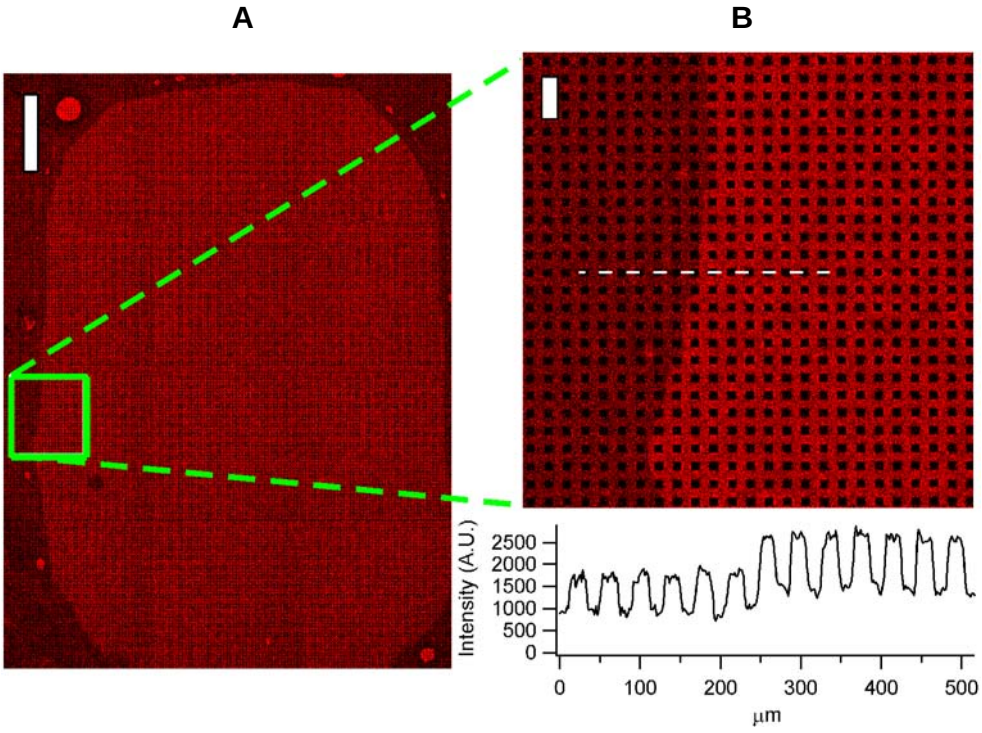


Figure 57 Fluorescence microscopy images of stamp coated with avidin-TR. The intensity line profile shows the average intensity in a 100 µm wide band along the white dashed line. Scale bars: (A) 1mm (B) 0.1mm.

The stamp surface contained a raised grid pattern consisting of 20 μm wide lines separated by 20 μm. A 200 μl drop containing 100 μg/ml avidin-TR was placed on the surface and spread out to cover the entire stamp. The drop immediately receded from the stamp edges. After a 10 min incubation 2 ml Millipore® water were added and the stamp was dried by spinning (2500 rpm 15 s). The 20 x 20 μm^2 wells were not wetted during the incubation and drying. Thus these areas were not coated with avidin-TR and appear black in the image. The intensity line profile clearly demonstrates a higher deposition of avidin-TR in the central part of the stamp.

Assuming that protein transfer from the stamp to the shim was complete and that the amount of protein transferred from the shim to the replica scaled with the amount of protein on the shim, we expected to observe a similar intensity distribution on in-mould patterned replicas. However, to our surprise, the intensity distribution was inverted. Figure 58 shows fluorescence microscopy images of a polypropylene replica in-mould patterned with avidin-TR. Avidin-TR was micro contact printed onto planar shims using stamps that contained the same relief structures and were coated in the same way as described above. Subsequently shims were mounted in the cooled mould and injection moulding was performed using polypropylene.

The intensity line profile clearly demonstrates that, contrary to what was observed on the avidin-TR coated stamp, there is a higher intensity and thus a higher protein density along the outer rim than in the central part of the patterned area on the replicas (Figure 58).

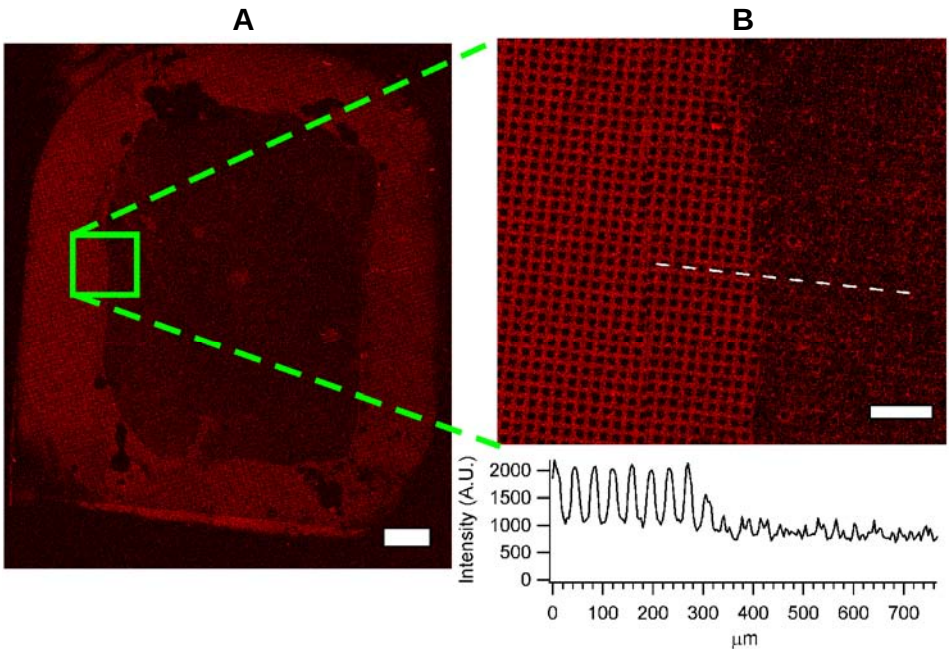


Figure 58 Fluorescence microscopy images of polypropylene replica in-mould patterned with avidin-TR. The intensity line profile shows the average intensity in a 100 μm wide band along the dashed white line. Scale bars: (A) 1 mm (B) 0.2 mm.

We therefore carried out a study of the protein transfer in in-mould patterning, tracking protein throughout the process by use of fluorescence microscopy. The study was performed using the fluorescence labelled avidin-TR, which allowed us to track protein transfer without the need for immunolabelling.

The use of fluorescence microscopy made it feasible to examine transfer over large areas (typically $1cm^2$). Fluorescence microscopy does however not give quantitative information on the amount of protein transferred. This is due to several factors such as: variations in the intensity of the laser used for excitation of the fluorophores between different measurements and variations in fluorescence quenching on shims and replica. Furthermore the large span in fluorescence intensity over the patterned areas makes it necessary to vary the gain of the detector when recording images. As the variation in detector output as a function of gain is not known this further prevents quantitative analysis of protein densities.

The data presented are from experiments carried out on FOx coated shims. An equivalent set of experiments was carried out on pure nickel shims. Due to substantial quenching of the fluorescence by nickel it was not possible to follow the process as was done for the FOx experiments. However, replicas produced from nickel shims showed the same pattern of transfer as was found when using FOx coated shims.

Flat shims were patterned with avidin-TR by micro contact printing. For this we utilized structured stamps containing a raised grid pattern consisting of 20 μm wide lines separated by 20 μm in each lateral direction. The use of structured stamps provided uncoated areas to be used as internal reference for determining the background intensity in the fluorescence microscopy images. The stamps were inked by placing a 200 μl drop containing 100 μg/ml avidin-TR on the surface of the stamp and spreading it out to cover the entire stamp. The drop immediately receded from the stamp edges. After 10 min incubation the stamp was mounted on a glass slide in a spincoater, 2 ml of Millipore® water were added and the stamp was spun dry (2500rpm, 15 s). Visible drops of water remaining on the stamp after spinning were removed with a tissue. In order to ensure complete transfer of protein in micro contact printing, the stamp should be brought in contact with the substrate within 1min after drying[26]. Therefore stamps were not imaged prior to micro contact printing but were immediately placed on the shim to be patterned. Fluorescence microscopy images were recorded of:

- Stamps after micro contact printing.
- Shims prior to injection moulding
- Replicas
- Shims after injection moulding.

Injection moulding transfer of the proteins was performed within 6 hours of the micro contact printing. In the time between micro contact printing and injection

moulding, shims were imaged in fluorescence microscopy and otherwise stored at room temperature in the dark. Injection moulding was performed using PP heated to 210^{o}C, injection speed of 204mm/s, 6s after-pressure, 66s cooling time and a mould cooled to 15^{o}C ± 1^{o}C.

5.1.1 Transfer efficiency from stamp to shim.

Fluorescence microscopy images of stamps after micro contact printing showed a high degree of protein transfer from stamp to shim in nearly all raised areas of the stamp (Figure 59 A). For areas placed in the central part of the stamp, the average intensity after stamping was 87 A.U. ± SD 6 A.U. after subtraction of background. Stamps that were coated using the same procedure but were not utilized for printing, showed an average intensity in the central part of the stamp of 2920 A.U. ± 238 A.U. after subtraction of background. This demonstrates a large degree of transfer in the micro contact printing. To avoid saturation of the detector, images of stamps not utilized for printing were recorded using a lower detector gain than the one used when imaging printed stamps. The actual difference between intensities before and after stamping is therefore significantly larger than indicated by these results, thus demonstrating an approximate transfer rate higher than 97%.

Considerable remaining intensity was found in a few isolated areas marked by white arrows in Figure 59 A. Confocal fluorescence microscopy showed that the bottom of the 20 x 20 μm^2 wells on the stamp were protein coated only in these areas. This, and the high intensity, suggests that drops of solution containing protein remained in these areas after the stamp was spin-dried. These drops then dried out leaving patches containing a large amount of protein, most likely considerably more than a monolayer. These protein patches were partly transferred in the micro contact printing since images of micro contact printed shims showed a large amount of protein transferred in the corresponding areas. Incomplete transfer also occurred in areas close to (1-2 mm) the edges of the stamp most likely due to lack of conformal contact between the stamp and the shim.

In conclusion, proteins are efficiently transferred from the stamp onto the shim in all areas except areas that are in the vicinity of the stamp edges and areas that show signs of large amounts of protein deposited on the surfaces due to drying of protein containing drops.

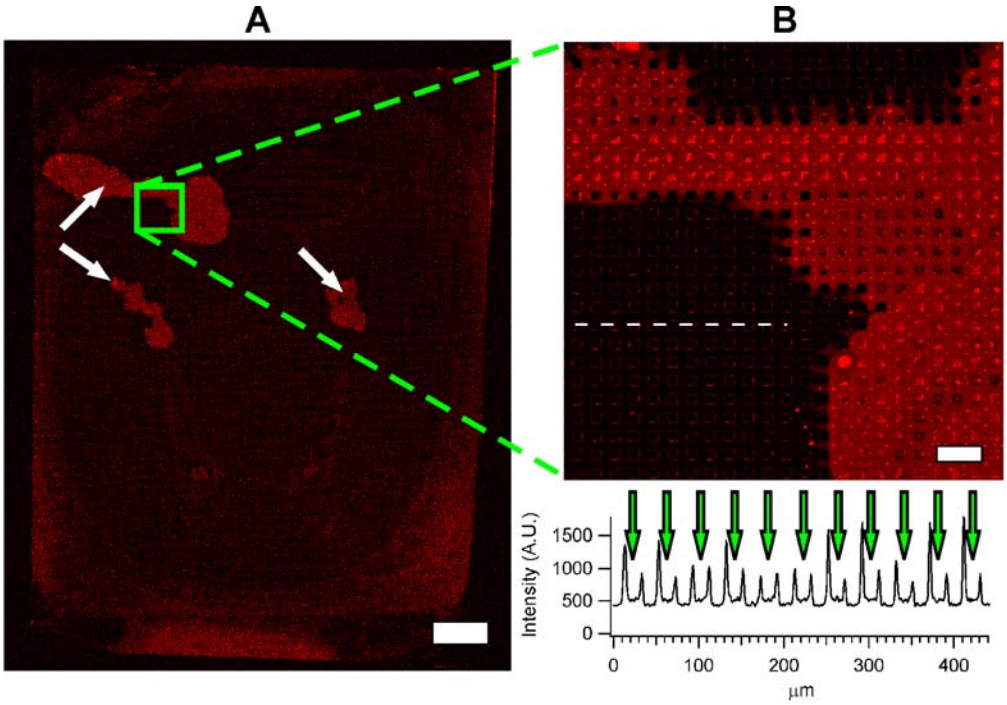


Figure 59 Fluorescence microscopy images of a stamp coated with avidin-TR and subsequently utilized for micro contact printing on a flat shim. (A) White arrows indicate areas where drops of solution have dried out leaving patches of protein that have only been partially transferred in the micro contact printing. Wells were fully protein coated only in these areas. (B) The intensity line profile shows the average intensity in a 10 µm wide band along the white dashed line. Green arrows indicate which parts of the curve correspond to raised areas on the stamp The distinct peaks stem from avidin-TR retained along the inner edges of the wells. Scale bars: (A) 1 mm (B) 0.1 mm.

5.1.2 Transfer from shim to replica

Fluorescence microscopy images (Figure 60) of identical areas on replicas and on shims prior to and after injection moulding were recorded. To avoid saturation of the detector when imaging the shims prior to injection moulding and to ensure detection of minor amounts of avidin-TR remaining on the shim after injection moulding, images were not recorded using the same detector gain. Therefore intensities cannot be compared directly.

Figure 60 presents a series of images following protein transfer in an easily recognizable area of the protein pattern. Intensity line profiles were extracted across identical paths on the protein pattern. If the replica intensity profile is plotted directly, the position of the observed peaks does not coincide with the position of peaks from images of the shim. This reduced distance between the protein features is likely to be due to shrinkage of the replica caused by thermal contraction of the polymer after injection moulding[85].The intensity line profile across the replica has therefore been plotted on an x-axis that is expanded 4% relative to the line profiles of the shim thus aligning the observed peaks. Images and profiles clearly show that transfer from shim to replica is far from complete

in all areas. Areas a, b and h, with intensities below 900 A.U. on the shim before injection moulding, were transferred completely in the injection moulding process. From areas c and d protein transfer was complete along the outer parts of the areas where intensities were below 1100 A.U. Markedly less protein was transferred from the central parts of these areas showing intensities above 1200 A.U. From areas e, f and g with intensities mainly above 1500 A.U. little or no transfer took place, apart from the outer 2-3 μm where intensities were lower.

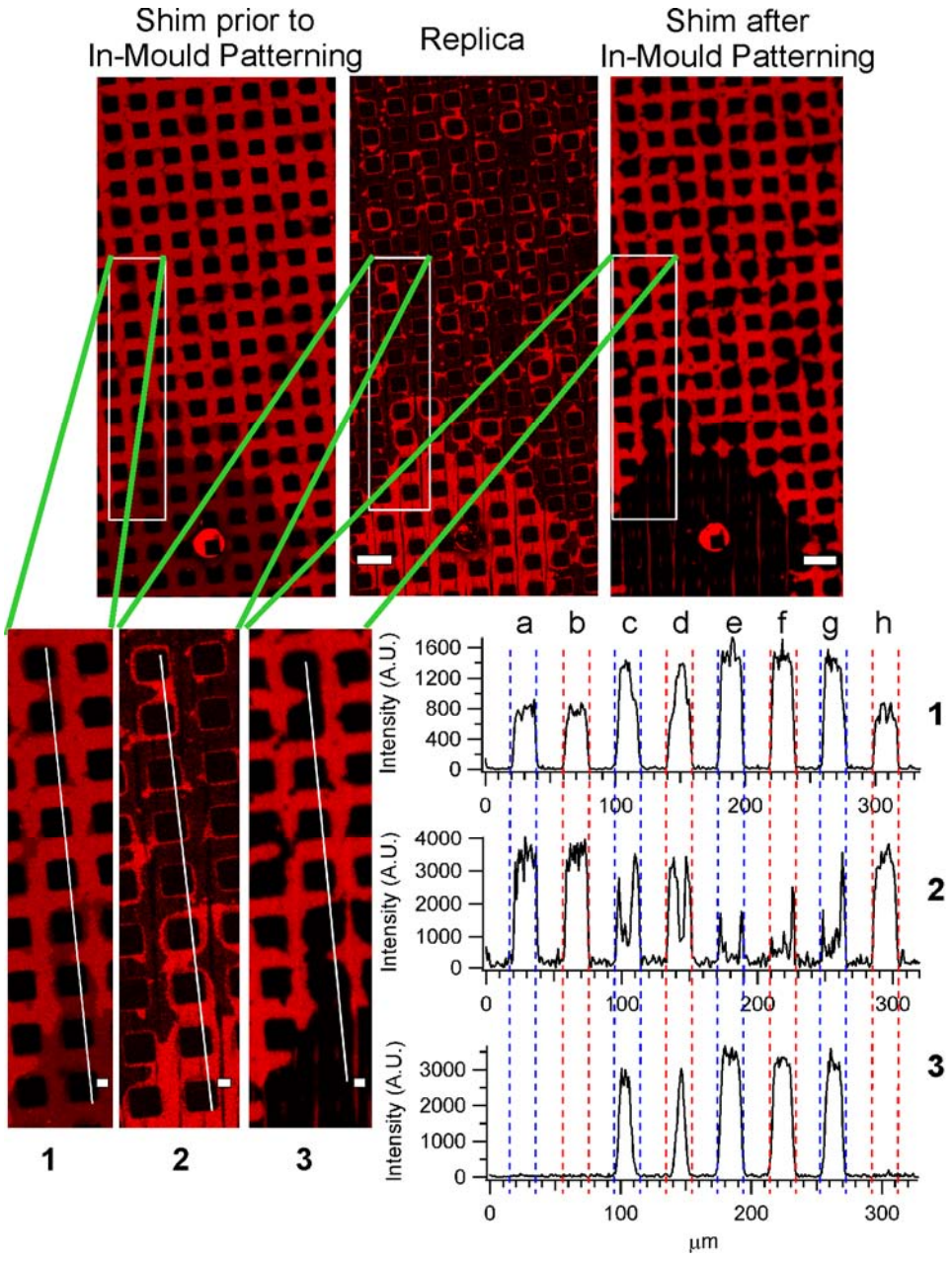


Figure 60 Fluorescence microscopy images depicting the same part of the protein pattern on the shim prior to injection moulding, on the replica and on the shim after injection moulding. Images of the replica have been mirrored in order to ease direct comparison with images of the shim. Line profiles show intensities along the paths indicated by the white lines. Scale bars: Upper part 50μm, lower part 10μm.

Protein transfer thus seems to be complete over a wide range of protein concentrations, here corresponding to intensities between approximately 40 and 1100 A.U. However, as the protein concentration is increased further, transfer falls off distinctly over an intensity span of approximately 200 A.U. Thus an increase in protein density of approximately 18% on the shim leads to a 77% decrease in protein density on the replica. As the protein density on the shim is increased further protein transfer decreases to near-zero transfer.

To confirm that this correlation between protein concentration and transfer was general we analysed intensities of a large number of matching areas on shims prior to injection moulding and the resulting replicas. This analysis was carried out on the three in-mould patterned areas shown in Figure 61.

Intensities were evaluated by extracting line profiles averaging intensities over a 10-100 μm wide band. We ascertained that averaging was performed over areas with homogeneous intensity distribution by confirming that peak intensities did not vary markedly when the width of the band for averaging was increased. Subsequently, the average intensity of the peaks was determined. Background intensities were determined from extracted line profiles without averaging of intensities (Figure 61).

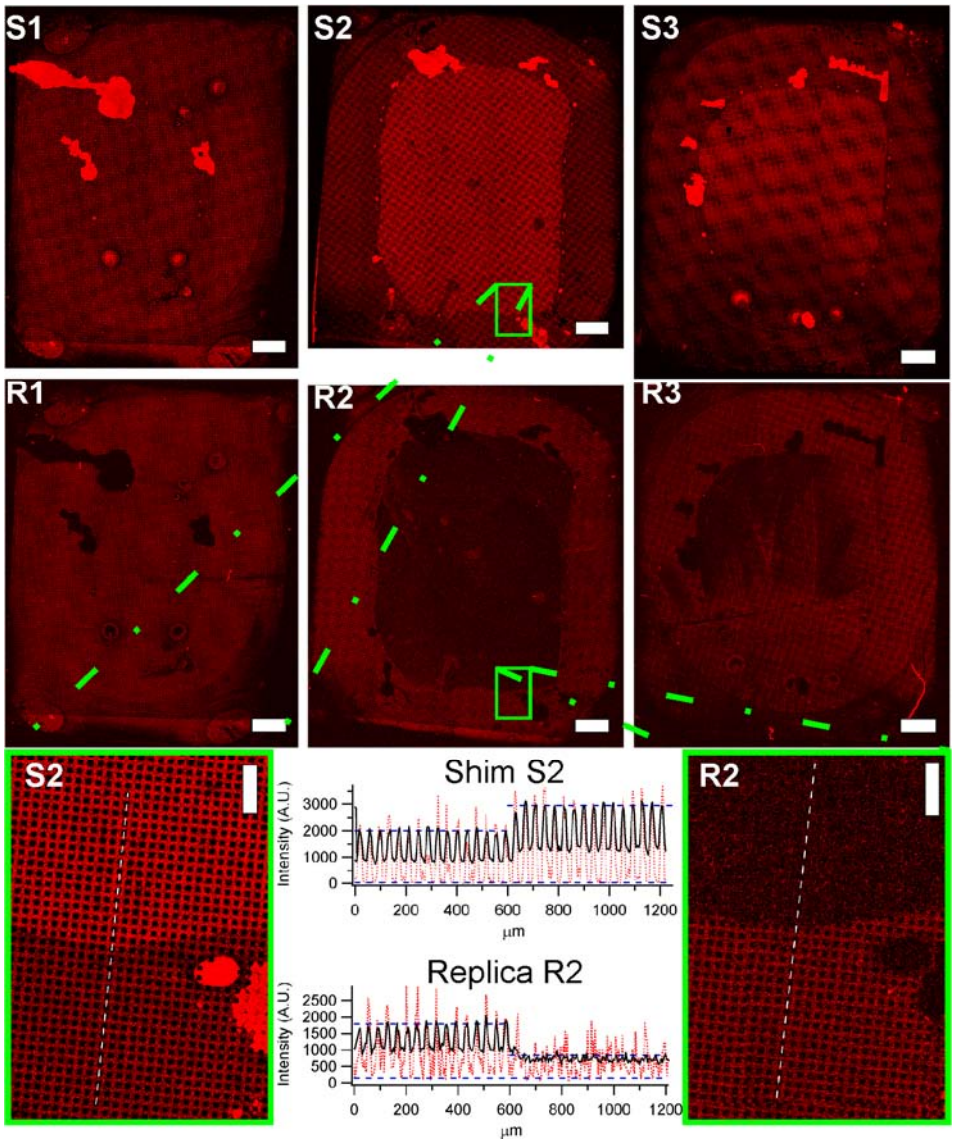


Figure 61 Fluorescence microscopy images of shims patterned with avidin-TR prior to injection moulding (S1, S2, S3) and the resulting polypropylene replicas (R1, R2, R3). All images were recorded using the same microscope settings. Solid black lines in the plots show the average intensities in a 50 µm wide band along the white dashed lines in the two enlarged areas. The dotted red lines in the plots show intensities along the same path without averaging. Horizontal blue lines in the plots mark the average peak intensities and the background intensity level. Scale bars: Upper and centre images 1000 µm. Lower images 200 µm.

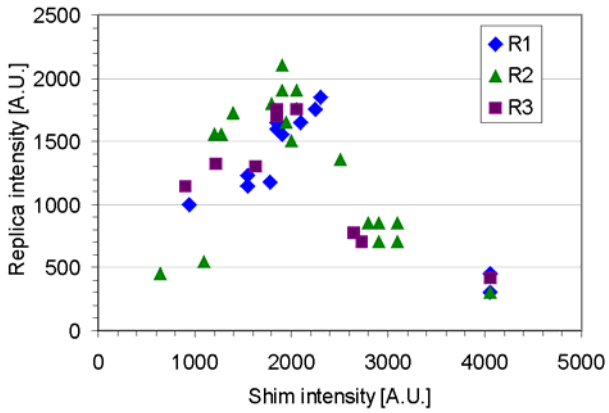


Figure 62 Average intensities measured on polypropylene replicas patterned with avidin-TR plotted versus the intensity measured in the corresponding areas on the shims prior to injection moulding.

Intensities on the three shims ranged from 650 to 4050 A.U.(detector saturation at 4050 A.U.) with the majority of high intensity areas placed between 2650 and 3100 A.U. Intensities above 3100 A.U. were measured only in areas where drops of protein solution had dried out leaving patches of high protein content.

In the intensity range between 650 and 2300 A.U. transfer increased with increasing protein density on the shim. As protein concentration was increased further, a sharp transition point was encountered and transfer decreased and continued to fall to a near-zero transfer (Figure 62). The intensity distribution on shim S1 contained intensities below 2050 A.U. in the majority of the central area. The complete transfer of protein in this area showed that the repeatedly observed incomplete transfer from the central part of the stamped areas was caused by high protein concentrations in this area leading to incomplete transfer.

It is clear that the correlation between the amount of protein on the shim and on the replica for all the three large examined areas (Figure 62) is identical to the correlation found when analysing the high-resolution images (Figure 60). This demonstrates that, within a wide range of protein densities, transfer is complete and it is thus possible to control the protein concentration on the replica directly by depositing the required density of protein on the shim.

5.2 Protein transfer mechanism

Disregarding areas where drops of solution dried out leaving patches with large amounts of protein, all areas that show near-zero transfer on shims S2 and S3 (Figure 61) have intensities within 2650 and 3100 A.U. This covers only 11% of the range of detected intensities on the shims. Shim S1 did not contain areas of intensity higher than 2300 A.U. and did not exhibit incomplete transfer. Additionally, no areas with intensities between 3100 and 4050 A.U. were found on any of the three shims (Figure 62). This indicates that in areas where

incomplete transfer was observed, a maximal protein density was reached. The amount of avidin-TR in the drop that was placed on the stamp during incubation was sufficient to coat the stamp surface several thousand times (approximating the shape of the adhered avidin-TR molecule to a cube). Considering this, and the fact that protein transfer was very efficient in the micro contact printing, it is likely that the conversion between complete and incomplete transfer corresponds to the conversion between a sub-monolayer and a full monolayer of protein covering the shim.

We have not performed a similar study for the other proteins that have been transferred using in-mould patterning. However, fluorescence images of replicas in-mould patterned with IgG/HRP and immunolabelled with IgG-FITC show the same patterns of higher protein concentration along edges of the stamped areas as was observed for avidin-TR (Figure 63). In these experiments stamps were also coated using a high protein concentration allowing for formation of a full monolayer on the stamp. This suggests that the observed drop in transfer when using high protein concentration is not specific for transfer of avidin.

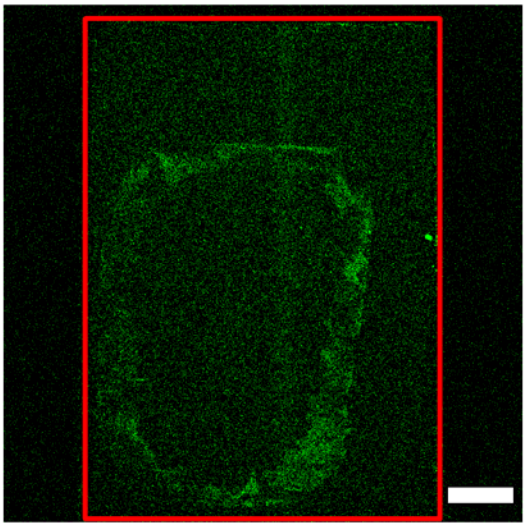


Figure 63 Fluorescence microscopy image of polypropylene replica in-mould patterned with IgG/HRP and immunolabelled using a fluorescence labelled antibody. The red square marks the borders of the well created around the stamped area by placing a PDMS mask on the replica. Scale bar 2 mm.

The mechanism behind protein transfer from the shim onto the replica is not clear. Proteins may adhere to the polymer melt as it contacts the proteins on the shim. The sudden drop in transfer may then be caused by less accessible surface area per protein, and thus weaker binding, as proteins are packed more tightly on the surface. Another mechanism for transfer may be partial encapsulation of proteins on the shim by the polymer melt. Upon solidification this would fix the proteins in the replica surface leading proteins to be pulled off the shim as the replica is separated from the mould. In this case tightly packed proteins on the shim surface would prevent this partial encapsulation thus causing the sudden drop in transfer (see illustration in Figure 64).

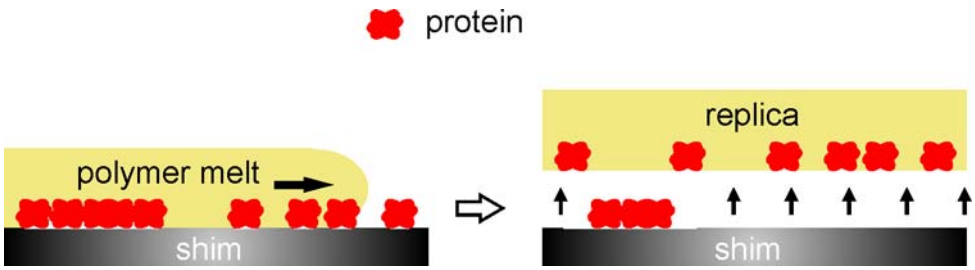


Figure 64 Sketch illustrating how transfer of proteins from shim to replica may occur. Proteins may be detached from the shim due to a stronger adhesion to the polymer than to the shim, or may be partially encapsulated by the polymer causing proteins to be ripped off the surface upon separation of the replica and the shim.

Both the suggested mechanisms could account for the observed correlation between increasing protein concentration and transfer. Therefore it is not possible, from the performed experiments, to determine which of these two mechanisms, if not both, contribute to the transfer.

The presented results provide a solution to a problem that was essential to solve, in order to preserve the main attraction of in-mould patterning i.e. the potential for low-cost mass production. Throughout our experiments we have performed a cleaning step between each injection moulding. This was done to ensure that no protein from earlier cycles remained on the shim. However, such a cleaning step would severely reduce the speed with which the products could be processed. As the presented results show the need for such cleaning can be avoided simply by controlling the amount of protein deposited on the shim. Furthermore, the complete transfer allows strict control over the protein density on the final part.

This emphasizes the need for establishing a reliable method to transfer precise submonolayer quantities of protein to the shims. One possible method for this could be using flat PDMS stamps as presented in section 3.6.1. Another approach could be to employ a technique recently developed by Mayer *et al.*[115]. Using stamps formed in agarose gel they were able to print 100 times using the same stamp without intermediate re-inking. Agarose stamps are likely to be too soft to avoid sagging when printing on the structured shim. They may, however, be utilized as inkpads for flat PDMS stamps thus avoiding the need for a washing and drying step entirely. As proteins adsorb onto a surface from solution when using agar stamps, controlling the stamping time and the concentration of the protein solution applied to the agar stamp can vary the protein concentration. Furthermore Mayer *et al* demonstrated patterning of several different proteins in one printing step[115]. Incorporating this or other multiple protein patterning approaches into the in-mould patterning process would allow production of e.g. microfluidic devices for one-step detection of multiple analytes.

5.3 Unspecific adsorption in patterned areas

If transfer is indeed impaired by formation of a monolayer of proteins on the shim, this means that the technique is limited to the transfer of sub-monolayers. For many applications this may make a subsequent blocking of the produced surfaces necessary in order to avoid unspecific adhesion of molecules from analyte solutions. Preliminary experiments to examine unspecific adsorption of proteins on in-mould patterned areas were performed using fibrinogen. This molecule is often examined when studying protein adsorption due to its high concentration in blood (3mg/ml)[116].

Using flat stamps, IgG-Alexa was micro contact printed onto the structured nickel shim. Stamps with a size of app. 1 cm^2 were inked by placing a 200 µl drop of a 25 µg/ml solution of IgG-Alexa on the stamp surface. After 10 min incubation the stamp was mounted on a glass slide in a spin-coater, 2 ml of Millipore® water were added and the stamp was spun dry (2500 rpm, 15 s). Immediately after drying, the proteins were micro contact printed onto the structured shim. After mounting the shim in the cooled mould, injection moulding was performed using polymethylmethacrylate. The replica was immersed in a solution containing 60 µg/ml fluorescence labelled fibrinogen from human plasma (Alexa fluor 488 fibrinogen conjugate from Molecular Probes)(Fibrinogen-488). After a 2 hour incubation at room temperature the replica was rinsed thoroughly in Millipore® water and dried in a stream of argon.

Figure 65 shows fluorescence microscopy images of a replica after incubation in fibrinogen. The area imaged is on the border between a flat area and an area containing 3.1 µm wide trenches separated by 10 µm. Intensity line profiles (Figure 65 C) show that, as expected, the amount of adsorbed fibrinogen-488 is lower in areas coated with IgG. Fibrinogen-488 adsorption decreases linearly with increasing amount of IgG-Alexa (Figure 65 D). The minimum amount of fibrinogen-488 adsorbed was calculated assuming that areas not coated with IgG contain a monolayer of fibrinogen-488 and correcting for background intensity that was measured prior to immersion in fibrinogen-488. The average intensity of the fibrinogen-488 signal measured in areas showing the highest density of IgG corresponds to a 90% ± 9% reduction in adsorption. This shows a significant reduction of adsorption in the patterned areas. However, the results also indicate that additional blocking may be necessary for applications that demand very high sensitivity.

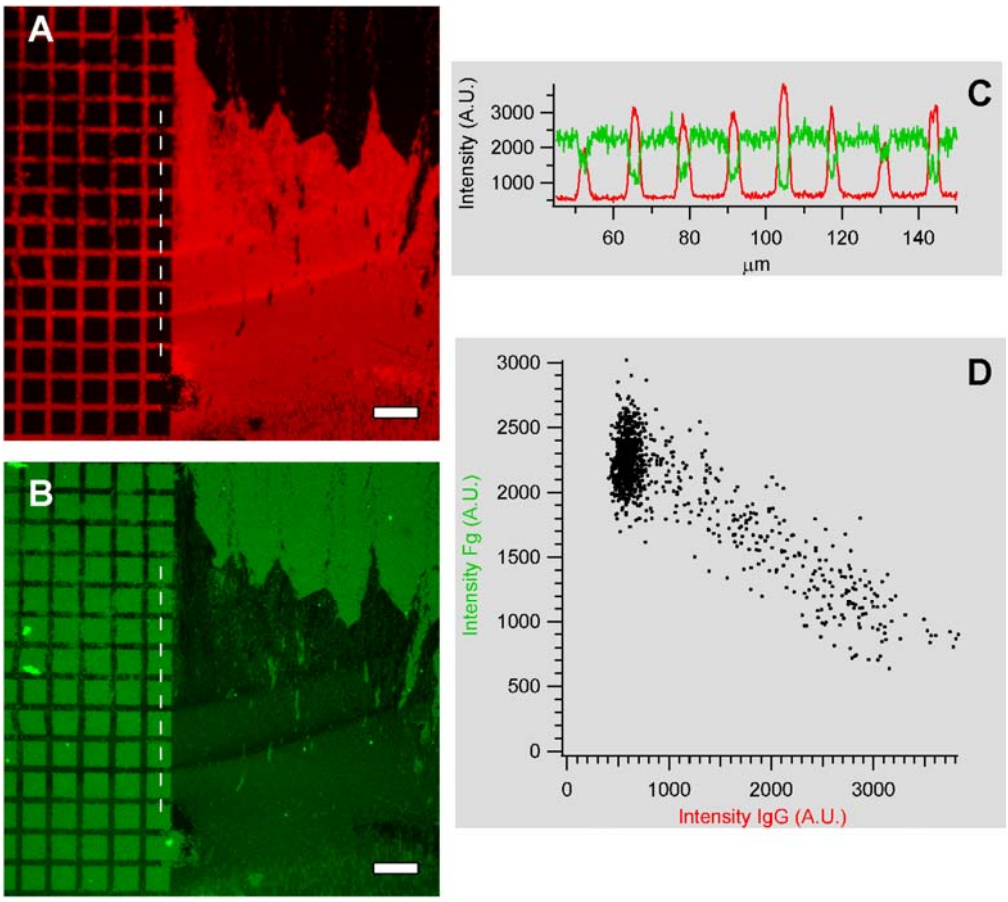


Figure 65 (A) (B) Fluorescence microscopy images of polymethylmethacrylate replica in-mould patterned with IgG-Alexa (red) and incubated with a solution containing fibrinogen-488 (green). (C) Line profiles show intensities along the two paths indicated by the dashed white lines. (D) Intensities of the fibrinogen-488 signal are plotted versus intensities of the IgG signal in the corresponding region. Scale bars are 20 µm.

5.4 Conclusion and outlook

This thesis presents the development of in-mould patterning, a novel technique for immobilizing patterns of proteins on polymer surfaces without the need for prior chemical modification of the polymer surface. Protein patterns with feature sizes spanning three orders of length scales were produced. There was no indication that the 100 nm size of the smallest features produced represents a lower limit to the feature size.

In-mould patterning combines the industrial production process of injection moulding with micro contact printing, a very popular technique, particularly in basic cell research, due to its flexibility, simplicity and low cost. Through micro contact printing, proteins were patterned on a mould and subsequent injection moulding immobilized the proteins on the polymer replica surface. In this way,

the method integrates the shaping of a polymer part with the formation of a protein pattern on the surface of the polymer part. Through using different combinations of a structured or flat mould and structured or flat stamps, complex patterns of proteins were produced. Moreover, we demonstrated that protein could be immobilized in recessed areas of the polymer surface. This can also be obtained using recently developed techniques, but often requires a multiple-step procedure[63,113] and has not yet been shown to produce as small feature sizes as presented here.

Most importantly this thesis work demonstrates that proteins transferred by in-mould patterning retain their biological function. The preservation of three different functionalities was tested. The enzyme horseradish peroxidase was in-mould patterned and retained catalytic activity of the transferred proteins was confirmed. Antibodies and avidin were in-mould patterned and were shown to retain the ability to bind their specific ligands. Finally, fibronectin, a cell adhesion promoting protein, was in-mould patterned on polypropylene and polystyrene surfaces and was shown to induce cell adhesion specifically in the patterned areas.

These results show that in-mould patterning may be applicable to a wide range of proteins. The established ability of transferred avidin to bind biotin is particularly valuable. The avidin-biotin system is highly relevant in applications where preservation of the native structure of the patterned proteins is essential to the performance of the device. The feasibility of transferring avidin makes it possible to pattern biotinylized proteins or biomembrane parts on surfaces from solution. This can be done after the in-mould patterning of avidin. In this way, proteins can be patterned entirely avoiding exposure to denaturing conditions.

As part of the development of the in-moulding patterning technique, fundamental studies on micro contact printing using hydrophobic stamps were undertaken. It was established that, by use of spin drying, flat stamps could be coated homogeneously. This was a prerequisite for utilizing micro contact printing for the protein patterning step in the in-mould patterning process. AFM studies of micro contact printed IgG, fibronectin and collagen revealed that at low protein densities protein networks are formed on the stamp surface. Formation of these networks is not related to protein function and are thus not physiological but rather a drying phenomenon.

The efficiency of the protein transfer from the mould to the shim was examined in detail. This revealed that, a broad range of protein densities can be applied to the mould and transferred completely from the mould to the polymeric replica. Upon increasing the protein density above a certain level, the transfer rapidly decreases to a near-zero transfer. We suggest that the rapid decrease in transfer is caused by the formation of a complete monolayer on the shim. The formation of a monolayer restricts the contact area between the polymer and proteins causing incomplete transfer. The presence of a wide density range, in which complete transfer occurs, allows tight control of the final protein density on the produced

polymeric part. This offers the possibility of tailoring the protein concentration to match the concentrations needed for different applications.

Most importantly, the complete transfer of protein from mould to replica will facilitate mass production as it eliminates the need for performing a cleaning step between each injection. The entire process can thus be automated by fitting the injection moulding machine with a suitable robot. Robots, performing complex tasks, are standard equipment on many commercial injection moulding production systems and the micro contact printing step can easily be performed by a robot, thus allowing rapid production of parts.

Apart from the short production-cycle time, one of the main reasons for the enormous success of injection moulding as a production technique is the broad range of available materials that can be injection moulded. In this thesis work polypropylene, polymethylmethacrylate, polystyrene and polycarbonate were successfully utilized for in-mould patterning. This strongly suggests that the in-mould patterning process is applicable to a wide range of materials.

Further work to be completed, before the in-mould patterning technique can be put into use, includes determining to which degree the biological function of transferred proteins is retained. The most relevant factor to control is the biological activity of the produced surfaces. It is therefore necessary to perform studies in order to determine how accurately surface activity correlates with the transferred density of molecules. For some applications further processing of the patterned surfaces may be necessary in order to avoid unspecific adsorption of proteins in unpatterned areas. For many applications the traditional blocking by human serum albumin may be sufficient. However, production of surfaces for long term culturing of arrays of cells are likely to require development of a technique for effectively blocking protein and cell adsorption in unpatterned areas. This will be necessary in order to preserve pattern fidelity over longer times.

In-mould patterning has further been demonstrated to be applicable to the patterning of catalytic colloidal particles, a process that can be utilized for forming electrical circuits in recessed areas of polymer parts.

Our results indicate that there may be a wide range of applications for in-mould patterning within the life sciences. Microfluidic immunoassay systems for one time use is one application that may in particular benefit from a low cost production method, such as in-mould patterning, since the introduction of such devices is highly dependent on product cost. Such devices will permit e.g. point-of care analysis, allowing the process of diagnosing many types of disease to become considerably faster. The demonstrated retained functionality of in-mould patterned antibodies and the very homogeneous intensity distribution found in the fluorescence signal from e.g. immunolabelled IgG/HRP suggest that the technique is very well suited to the production of such devices. Incorporation of printing techniques allowing for patterning of more than one protein at a time will further increase the usefulness of the technique.

Although much work needs to be done to characterize the in-mould patterning process in full, we believe that the technique has the potential to deliver a number of the qualities sought after in order to move many of the emerging new technologies from the lab-bench into the production hall and thus into our daily lives.

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7 Appendix A

7.1 Abbreviations list / glossary

ABTS:2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt substrate from Sigma-Aldrich
AFM: Atomic Force Microscopy
Anti-FN:Monoclonal anti-fibronectin antibody produced in mouse fromSigma-Aldrich
Aspect ration: width of structure / height of structure
Avidin-TR: Avidin Texas Red conjugate from Sigma-Aldrich
Basal lamina: "The basal lamina(often erroneously called basement membrane) is a layer on shich epithelium sits. This layer is composed of an electron-dense layer(lamina densa) between two electron-lucid layers(lamina lucida), and is approximately 40-50 nm thick(with exceptions such as the 100-200 nm glomerular basement membrane). The lamina densa is composed of type IV collagen. The lamina lucida is adjacent to the epithelial cells and contains the glycoprotein laminin." {102}.
Biotin: Biotin-4-fluorescein Biotin-4-fluorescein from Sigma-Aldrich
D-PBS: Dulbecco´s Phosphate Buffered Saline
ELISA: Enzyme-Linked ImmunoSorbent Assays
Fibronectin: Fibronectin from human plasma from Sigma-Aldrich
FOx: Flowable oxide e-beam resist FOx-14 from Dow Corning
HaCat: HaCaT human keratinocyte cell line (DKFZ, Heidelberg, Germany)
HRP: Horseradish peroxidase
HSA: Human serum albumin Fraction V from Sigma-Aldrich
IgG-Alexa: Alexa Fluor 546 goat anti-mouse IgG (H+L) from Molecular Probes
IgG/HRP: Polyclonal goat anti-mouse immunoglobulin G/HRP from Dako Cytomation
Millipore® water:Millipore Milli-Q Academic from Millipore
MAPS: Micro stamping on Activated Polymer Surfaces
PBS: Phosphate Buffered Saline
PC: Polycarbonate
PDMS: Polydimethylsiloxane
PMMA: Polymethylmethacrylate Diakon TD 525 Lucite
PP: Polypropylene Inspire H715-12 from Dow Plastics
PS: Polystyrol 158K from BASF

8 Appendix B

8.1 Article submitted to Science

Risø's research is aimed at solving concrete problems in the society.

Research targets are set through continuous dialogue with business, the political system and researchers.

The effects of our research are sustainable energy supply and new technology for the health sector.